The role of Myeloid Hif-1α in Acute Lung Injury

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For my wife, son and parents

Timeo danaos et dona ferentes.

Declaration

I declare that all the work presented in this thesis is my own and has been performed by myself.
This work has not been submitted for any other degree or qualification.

Andrew MacDuff

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I would like to thank Dr Nik Hirani my supervisor for his help and support throughout the time when this thesis was being undertaken. I should also like to thank Dr Paul Fitch and Professor Sarah Howie for their freely given and invaluable help and friendship during my time.
To my wife and parents, I hope that the result excuses the means.

"And if I claim to be a wise man, it surely means that I don't know"
Kansas- Carry on Wayward Son.

List of abbreviations

ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
ARNT	Aryl hydrocarbon Receptor Nuclear Translocator
ATP	Adenosine 5'-triphosphate
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
cAMP	cyclic Adenosine Monophosphate
CBP	CREB binding protein
CIR	Centre for Inflammation Research, Edinburgh University
COOH	Carboxyl
CREB	cAMP response element-binding
CSF	Colony Stimulating Factor
DAB	Diaminobenzidine
DMOG	Dimethyloxallyl Glycine
DNA	Deoxyribose Nucleic Acid
EB	Evans Blue
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPO	Erythropoietin
FIH	Factor Inhibiting Hif
FiO2	Fraction of Inspired Oxygen
GLUT-1	Glucose transporter 1
Hif	Hypoxia inducible factor
HLF	Hif-Like Factor
HLH	Helix Loop Helix
HO	Heme Oxygenase
HRE	Hypoxia Response Element
HRF	Hif-Related Factor
HS	Haemorrhagic Shock
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
i.t.	Intratracheal
Km	Michaelis Constant
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide/Endotoxin
M	Molar
MAPK	Mitogen Activated Protein Kinase
mM	milli Molar
MODS	Multiple Organ Dysfunction Syndrome
MOF	Multiple Organ Failure

MOP2	Member of the PAS superfamily 2
MPO	Myeloperoxidase
NF-κB	Nuclear Factor κB
NH_2	Amino
NLS	Nuclear Localisation Signal
NO	Nitric Oxide
nNOS	Neuronal Nitric Oxide Synthase
ODD	Oxygen Dependent Domain
p300	E1A binding protein p300
PAS	Per/ARNT/Sim
PaO2	Arterial Partial Pressure of Oxygen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHD	Prolyl Hydroxylase
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear leukocyte/Neutrophil
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDF-1	Stromal Derived Factor-1
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
TAD	Transactivation Domain
TNF	Tumour Necrosis Factor
Ub	Ubiquitin
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau protein
W/D	Wet/Dry

Abstract

Acute Lung Injury, characterised clinically as the Acute Respiratory Distress Syndrome is a catastrophic response to a range of pulmonary and non-pulmonary insults. Despite much work the key mechanisms involved in generating the exaggerated immune response that results in lung injury are not completely understood.

Hypoxia-inducible factor-1 has been shown to be a key transcription factor in the myeloid cell response to inflammatory signals.

The aims of this thesis were to develop a model of acute lung injury and to study the role of Hif-1 in the generation of lung injury in this model.

A model of direct pulmonary injury as a result of intratracheal instillation of endotoxin is described. Using this model the role of myeloid cell Hif-1α was characterised using a myeloid cell specific conditional knockout system.

The injury in Hif-1α deficient mice was quantitatively similar to the injury seen in wild type animals over a range of time points. However, the quality of the injury, assessed by a measure of nitric oxide mediated damage was reduced.

The *in vivo* data were supported by *in vitro* studies using a murine macrophage cell line which showed that manipulation of the cellular oxygen tension in the presence of endotoxin alters the ability of the cell to generate nitric oxide. Furthermore, pharmacological manipulation of cellular Hif-1 levels by Dimethyloxallyl Glycine (DMOG) in the macrophage cell increased the generation of nitric oxide in response to endotoxin by altering the expression of a number of the isoforms of Nitric Oxide Synthase.

In a final set of experiments the response to intratracheal endotoxin was modulated in mice by the concurrent administration of DMOG. As expected the qualitative

response to endotoxin was similar but the NO mediated damage was enhanced in the animals administered DMOG.

Manipulation of Hif-1 may have a role in the therapy of lung injury by altering the characteristics of the response.

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Chapter 1

Introduction

1.1 Acute Lung Injury and the Acute Respiratory Distress Syndrome

1.1.1 Definitions

Multiple organ failure (MOF) is the leading cause of death in Intensive Care Units. The Acute Respiratory Distress Syndrome (ARDS), first described by Ashbaugh et al [1] in 1967 is the pulmonary, and often first, manifestation of this systemic inflammatory response. The body responds to a diverse range of insults in a stereotyped manner clinically recognisable as the Systemic Inflammatory Response Syndrome[2]. This constellation of findings: Body temperature greater than 38C or less than 36C; heart rate greater than 90 beats per minute; tachypnoea (respiratory rate greater than 20 breaths per minute or PaCO2 less than 32mmHg) and a white cell count greater than $12x10^6$ cells/ml or less than $4x10^6$ cells/ml, represents the clinical features of a systemic inflammatory process[2].

The study of ARDS was initially hampered by a lack of agreement on the clinical features of the disease and this resulted in confusion and inconsistency for both clinical care and scientific investigation. An American and European Consensus Conference (AECC) in 1992 attempted to resolve this confusion by establishing a clinical definition of ARDS consisting of 4 criteria: 1. acute onset of disease; 2. ratio of PaO2 to FiO2 of less than 200 (regardless of level of positive end-expiratory pressure); 3. bilateral infiltrates on a frontal chest radiograph (figure 1-1); and 4. pulmonary artery wedge pressure of less than 18mmHg or no clinical evidence of left atrial hypertension[3]. The term Acute Lung Injury was also introduced comprising

patients exhibiting the above signs but with a PaO2:FiO2 of less than 300. As the pulmonary manifestation of SIRS the onset of ARDS is often the event precipitating admission to intensive care and heralds the development of the Multiple Organ Dysfunction Syndrome (MODS)[2] reflecting a global loss of the body's normal homeostatic mechanisms.

Figure 1-1 Chest Radiograph of patient with ARDS showing bilateral alveolar infiltrates

The AECC definition has been found to be useful both in clinical practice allowing physicians to identify the process with easily available data and in clinical studies giving an identifiable patient group in whom to apply novel therapies. However, it must be appreciated that the AECC criteria are imperfect. When clinical and autopsy diagnoses are compared it has been found that the AECC criteria have only a moderate ability to identify both cases and non-cases (sensitivity 75%, specificity 84%[4]). Furthermore, there is only limited agreement between alternative diagnostic

criteria (lung injury severity score [5] and a Delphi definition [6]) [7]. It is also only recently being appreciated that different precipitating insults may result in differing clinical presentations [8;9] suggesting that future work may need to divide patients according to the suspected primary insult (although this may be complicated in the critically ill patient who has a degree of sepsis, may have aspirated as a result of a reduced level of consciousness and has received blood products as part of goal-directed therapy[10]). Despite these limitations however, the AECC criteria have allowed the identification of a group of patients who, whatever the exact underlying pathology, benefit from a specific ventilatory targets[11] (see section 1.1.5 Therapy of ALI/ARDS). Also, the criteria recognise that the severity of lung injury varies (ALI versus ARDS) and this may facilitate earlier identification of patients and earlier application of interventions to prevent deterioration.

1.1.2 Epidemiology and physiology of ALI/ARDS

ALI can be the result of direct (most common) or indirect insults to the pulmonary system. (Table1-1). The most common direct injury is pulmonary sepsis and the most common indirect insult is sepsis. The incidence is variably reported depending on the intensive care unit setting and patient population but an incidence of 20-50 per 100000 person years is widely accepted[12]. The mortality is approximately 40-50% but ranges from 30-74% again depending on case mix[12]. Most deaths do not occur as a result of respiratory failure (less than 20% of deaths) but rather as a result of MODS reflecting the systemic nature of the pathological process. The primary physiological problem in ALI is severe hypoxaemia associated with a marked increase in minute ventilation as a result of a sharp increase in pulmonary deadspace [13]. There is also a decrease in lung compliance with an associated increase in the work of

breathing. This combination of factors results in refractory respiratory failure usually necessitating intubation and positive pressure ventilation with high fractions of inspired oxygen to achieve arterial oxygenation approaching satisfactory levels.

Table 1-1 Causes of Acute Lung Injury

Direct Pulmonary Insult	Indirect Pulmonary Insult
Pneumonia	Sepsis
Aspiration	Multiple Trauma
Inhalation Injury	Cardiogenic shock
Pulmonary Contusion	Acute Pancreatitis
Fat embolism	Cardiopulmonary bypass
Drowning	Disseminated intravascular coagulation
Reperfusion Injury	Burns
	Neurogenic Pulmonary Oedema
	Transfusion related lung injury

1.1.3 Pathology of ALI/ARDS

The histological hallmark of ARDS is Diffuse Alveolar Damage (DAD) [14;15]. DAD is characterised by a sequential series of pathological change that occur following the injurious stimulus[16]. DAD is the result of severe damage to the alveolar-capillary unit[17].

There are 3 recognised histological phases: Early, exudative phase; proliferative phase and fibrotic phase. These phases often overlap and can co-exist in different parts of the same patient's lung. The phases are not necessarily progressive as injury can stop and recovery occur at any time.

Exudative phase

This phase occurs in the first week following injury and is an acute inflammatory response accompanied by a marked influx of neutrophils resulting in injury to the alveolar epithelium and vascular endothelium[17]. At necroscopy the lungs are heavy, rigid and dark. Microscopically there is extensive alveolar and interstitial oedema with hyaline membranes containing immunoglobulin and complement. The microvascular and alveolar barriers are damaged with necrosis of alveolar cells. The necrosis of type I epithelial cells results in a breakdown of the gas exchange and barrier function of the lung which results in flooding of the air spaces with protein rich oedema fluid. Endothelial cells swell leading to widening of cellular junctions and disrupted barrier function [16]. There is an intense predominantly neutrophilic infiltration which is initially seen in the interstitium but extends to involve the airspaces[15]. BAL samples show an expanded alveolar neutrophil population [18]. Electron microscopy studies show endothelial injury (cell swelling, widened

intercellular junctions and increased pinocytotic vesicles) with damage to type I and II epithelial cells [19]. The components of the hyaline membranes are mainly derived from damaged epithelium, their secretory products and some serum proteins. The proliferative phase begins from approximately day 3 onwards.

Proliferative Phase

This second stage of DAD is characterised by type II alveolar epithelial cell hyperplasia and proliferation with relining of the denuded basement membrane. There is an ingrowth of mesenchymal cells and the formation of intra-alveolar organised granulation tissue [16]. Type II pneumocytes migrate over the surface of the granulation tissue transforming the intra-alveolar exudates into interstitial tissue. Called fibrosis by accretion, this is the most important mechanism of remodelling in DAD[16]. The end result is of fewer but larger alveoli with more dilated alveolar ducts [19]. Macroscopically the lungs are now densely consolidated with re-brown or yellow-grey discolouration.

Fibrotic phase

Fibrosis can begin within 36 hours of injury but the peak of this phase is seen after 3 to 4 weeks. There is thickening of the alveolar septa due to collagen deposition with few fibroblasts. There are also vascular lesions with mural fibrosis and thickening of small muscular arteries, veins and lymphatics. Macroscopically there is extensive lung remodelling with alternating areas of microcysts and irregular scarring. However, not all patients develop fibrosis [15] for reasons that are not understood. Indeed, if the patient survives the event the long term sequelae tend to reflect the neurological effects of critical illness (Critical Illness Polyneuropathy)[20] rather than impaired pulmonary function.

1.1.4 Pathogenesis of ALI/ARDS

The pathogenesis of ALI/ARDS is incompletely understood despite much investigation[15]. A recent consensus conference on behalf of the National Heart, Lung and Blood Institute in the United States emphasised the significant lack of knowledge and understanding of the pathological processes seen in these critically ill patients [13]. A recent review[21] has summarised the current understanding of the interplay between alveolar macrophages (acting as sensing and directing cells), neutrophils, alveolar epithelium type I and II cells and pulmonary endothelium (fig 1.2).

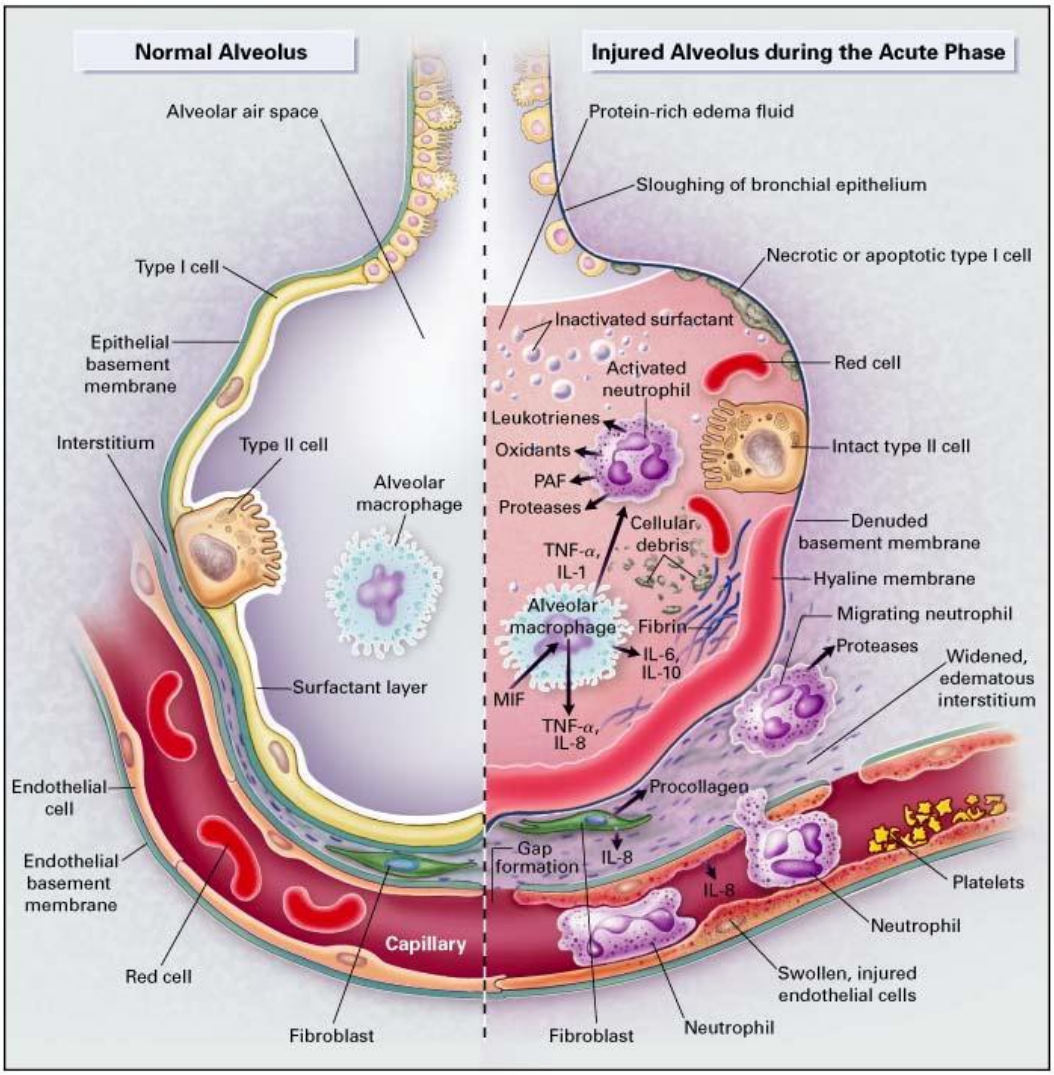


Figure 1-2 Summary of cellular interactions during the acute phase of lung injury.

In the acute phase of the syndrome (right-hand side), there is sloughing of both the bronchial and alveolar epithelial cells, with the formation of protein-rich hyaline membranes on the denuded basement membrane. Neutrophils are shown adhering to the injured capillary endothelium and marginating through the interstitium into the air space, which is filled with protein-rich oedema fluid. In the air space, an alveolar macrophage is secreting cytokines, interleukin-1, 6, 8, and 10, (IL-1, 6, 8, and 10) and tumour necrosis factor α (TNF-α), which act locally to stimulate chemotaxis and activate neutrophils. Macrophages also secrete other cytokines, including interleukin-1, 6, and 10. Interleukin-1 can also stimulate the production of extracellular matrix by fibroblasts. Neutrophils can release oxidants, proteases, leukotrienes, and other proinflammatory molecules, such as platelet-activating factor (PAF). A number of anti-inflammatory mediators are also present in the alveolar milieu, including interleukin-1–receptor antagonist, soluble tumour necrosis factor receptor, autoantibodies against interleukin-8, and cytokines such as interleukin-10 and 11 (not shown). The influx of protein-rich oedema fluid into the alveolus has led to the inactivation of surfactant. MIF denotes macrophage inhibitory factor. Modified from ref [21]

As a result of one or often several insults (the so called two hit hypothesis where the first insult primes the body to initiate an exaggerated disproportional response to a second event) an uncontrolled inflammatory response is generated which results in neutrophil recruitment and the generation of alveolar-capillary damage.

There has been much effort expended in trying to identify a single, key mediator responsible for the development of the characteristic features of ARDS. However, it would seem increasingly clear that this approach is over simplistic and that the disease is the result of a complex interaction of a range of mediators and effector cells which may vary between differing clinical precipitants [8;9]. However, a number of mediators (see Table 1-2) are consistently identified as being of importance in the generation of ARDS.

Table 1-2 Mediators involved in ARDS

Inflammatory Mediator	*Function*
TNF-α	Pro-inflammatory, Neutrophil activation
IL-1β	Pro-inflammatory, Neutrophil activation
IL-6	Leukocyte activation, Pyrexia
Chemokines including IL-8	Leukocyte activation and chemotaxis
IL-10	Anti-inflammatory
Platelet activating Factor	Leukocyte activation, platelet activation
Reactive oxygen and nitrogen species	Regulation of vascular tone, antimicrobial

TNF-α and IL-1β

Tumour necrosis factor-α and Interleukin-1β are predominantly derived from activated macrophages and act via specific receptors. Both are present in the bronchoalveolar lavage fluid and plasma of patients at risk of and with ARDS[22] with an alveolar-plasma gradient suggesting a pulmonary source. Both are pro-inflammatory capable of activating inflammatory cells. However anti-TNF-α[23] or IL-1β[24] therapeutic strategies have been unsuccessful in clinical trials suggesting

that either other mediators have more important roles or that the therapeutic window is missed in these trials[25].

Chemokines including IL-8

Chemokines are small cytokines that bind specific G-protein receptors[26]. They function in leukocyte trafficking, recruitment and re-circulation. Chemokines are present in the BAL of ARDS patients [26]. IL-8 levels correlate with the severity of neutrophil infiltration in ARDS. The neutrophil chemotactic activity of ARDS BAL has been shown to be predominantly due to IL-8[27;28]. Early neutrophil recruitment to lungs mediated by IL-8 may be a key feature in the pathogenesis [29].

Interleukin-6

IL-6 is produced by a wide range of cells including monocytes/macrophages, endothelial cells and fibroblasts[22]. Circulating levels of IL-6 have been shown to predict the severity of ARDS of different aetiologies including pancreatitis[30] and sepsis[31].

Interleukin-10

IL-10 is an anti-inflammatory cytokine which inhibits the release of pro-inflammatory cytokines such as IL-1 and TNF. IL-10 has been shown to inhibit alveolar macrophage production of these mediators and clinical studies have found reduced levels in patients who had ARDS compared to than those at risk of ARDS[32] but without evidence of clinical disease. This emphasises the importance of appreciating that it is likely to be the balance between pro- and anti-inflammatory mediators in ARDS rather than absolute levels that determines development of disease[25].

Neutrophil products including Elastases and reactive oxygen species

Neutrophils are effector cells in the development of pulmonary damage in ARDS. The uncontrolled release of neutrophil elastase and matrix metalloproteinases by neutrophils results in cell injury. Neutrophil elastase compromises the integrity of the alveolar-capillary barrier and can cleave exudate proteins such as fibrin into potent chemotactic peptides further driving the inflammatory process[33].

Neutrophils (and alveolar macrophages and epithelial cells) can produce reactive oxygen and nitrogen species[22] which have injurious effects on both alveolar epithelium and endothelium. Oxidant stress increases endothelial permeability and impairs the epithelial transport of sodium ions. Blockage of the synthesis of these species has been shown to be protective in a model of ARDS[34]. However, although important in the generation of lung injury the neutrophil is not necessary for its occurrence. ARDS is recognised to occur in profoundly neutropenic patients following bone marrow transplantation[35]. Hence other mechanisms including, but not limited to macrophage released mediators, subsequent epithelial and endothelial dysfunction, and microvascular thromboses are likely to be of importance [13] in the generation of lung injury.

1.1.5 Therapy of ALI/ARDS

Despite much effort and the insights gained into the pathophysiology occurring, therapy for these patients remains suboptimal. The mainstay of therapy is supportive care with invasive ventilation, adequate nutrition, renal replacement therapy if necessary and treatment of superadded infection. Traditional anti-inflammatory

therapy using steroids have shown no survival benefit [36;37]. Other possible interventions include inhaled Nitric oxide which, while improving oxygenation acutely, does not improve survival [38;39]. In addition prone positioning [40] and surfactant replacement [41-43] have proven ineffective. Potential biological therapies such as TNFα manipulation are complicated by the difficulties in reconciling an initial injurious effect of TNFα with a later reparative effect seen experimentally where both anti-TNFα therapy and TNFα administration have resulted in improved outcomes[44;45].

Currently the only intervention known to improve outcome is a lung protective ventilation strategy using a low tidal volume (less than 7ml/kg body weight as opposed to the traditional 10-15ml/kg)[11]. A recent Cochrane review has confirmed that this approach improves 28 day mortality[46]).

Therefore there is a need for an improved understanding of both the cellular and molecular events to help identify new therapeutic strategies.

1.1.6 Previous work leading to this study

An important early observation in the study of ARDS was that the BAL level of key cytokines (IL-8) was increased in trauma patients at risk of developing ARDS before radiological evidence was apparent [29]. This suggested that the critical insults occurred before the development of clinically detectable disease and that the pathological cellular activation and signalling must also occur early. The search for a possible universal initiating insult has been hampered by the diverse range of conditions that can result in ALI/ARDS. Furthermore, identification of the timing and

sometimes indeed, the exact nature of the insult that leads to ARDS can be difficult in many patient groups.

However, trauma patients generally have a recognised time and mechanism of insult. In clinical series of trauma patients hypoxia and hypotension are commonly seen [47]. Measures of the severity of global tissue hypoxia (magnitude and duration) in these patients predict the development of ALI and MODS [48;49]. Surgical patients present a group at risk of ALI in whom the nature of the insult is stereotyped and the timing and severity of physiological derangement are known. A group of surgical patients particularly at risk of developing ALI/ARDS are those undergoing thoracotomy. It has been estimated that about 5% of lobectomy/pneumonectomy patients develop ALI[50], termed postoperative lung injury (or postpneumonectomy pulmonary oedema in the USA)[51]. Similarly to ARDS in other groups the mortality associated with this injury is high (50-100%[51]). It has been recognised at autopsy that the pathological changes in the lungs are identical to those seen in ARDS of other causes[52]. Thoracotomy requires collapse and re-expansion of the operative lung to allow hilar dissection and pulmonary resection. Hence in addition to damage sustained during surgical manipulation, the operative lung is exposed to a period of hypoxia while the lung is collapsed followed by mechanical distension and hyperoxia when ventilatory recruitment manoeuvres are used to re-expand it. The non-operative side is also exposed to hyperoxia and, using traditional ventilatory patterns, mechanical distension during the period of one-lung ventilation. All of these factors singly or in combination could be the trigger for ALI in these patients.

Further clues to the triggering stimuli can be found in patients who undergo oesophagectomy. These patients undergo a thoracotomy in association with

abdominal dissection however there is no pulmonary resection and are at high risk of ALI/ARDS [53;54]. Oesophagectomy patients have higher serum IL-6 levels post-operatively compared to patients who have undergone surgery of a similar duration limited to the abdomen but with greater blood loss (pancreaticoduodenectomy) suggesting that the thoracotomy itself is linked to the severity of the inflammatory insult[55]. In these patients perioperative hypoxia is associated with the development of ALI [53]. Rocker et al [56] found that the thoracotomy side had a greater increase in lung permeability than the non-operative side, suggesting that local pulmonary insults are involved in the generation of the systemic changes. Kooguchi et al [57] have subsequently shown that there is an increase in BAL cytokines (IL-6 and IL-8) in these patients and that the alveolar macrophage is the main source of these. Furthermore subsequent analysis of Donnelly's original data [58] suggested that cytokine liberation was correlated with the severity of hypoxia. Kooguchi et al [57] have also shown that the intensity of macrophage cytokine expression correlates with measures of hypoxia.

Re-expansion pulmonary oedema is a potentially fatal condition that can be seen after rapid re-expansion of a collapsed lung as a result of treating a pneumothorax or pleural effusion. The collapsed lung, following a period of hypoxia, is rapidly reexpanded and the alveoli are exposed to inspired oxygen. Nakamura et al [59] have shown in a rabbit model that alveolar macrophages in the hypoxic lung secrete IL-8 before re-expansion and that treatment with a monoclonal anti-IL-8 antibody reduces the severity of the lung injury again linking alveolar hypoxia to lung injury mediators.

Finally, high altitude pulmonary oedema occurs in mountaineers who are exposed to alveolar hypoxia as a result of the decrease in partial pressure of oxygen at high

altitudes. Studies have shown that these patients have increased BAL inflammatory cells[60] and cytokines (increased IL-1β,-6,-8 and TNFα) [61], although these are not universally found [62].

Using both animal models and in vitro studies the potential pro-inflammatory nature of hypoxia has been recognised. Ertel et al [63] showed that in mice exposed to hypobaric hypoxia the plasma TNF-α and IL-6 were elevated and that peritoneal macrophages isolated from hypoxia exposed animals produced greater quantities of TNF-α, IL-1 and IL-6 in response to endotoxin stimulation in vitro. Metinko et al[64] have shown that human monocytes exposed to anoxia/reoxygenation secrete IL-8 and that in response to endotoxin the response is further exaggerated. In rats normobaric hypoxia alone has been found to induce pulmonary inflammation in the absence of any other insult [65] with features similar to ALI. Furthermore hypoxia has been shown to affect neutrophil function delaying apoptosis [66] and enhancing cytotoxic function [67]. Therefore, an early event such as pulmonary regional hypoxia which occurs before the development of ALI (as a result of pulmonary contusion in trauma, consolidation in pneumonia or atelectasis in critically ill patients) is a potential stimulus for the development of lung injury.

Other stimuli such as hyperoxia [68] and mechanical stretch during mechanical ventilation [69;70], both of which are used to treat ARDS, can undoubtedly contribute to the development of ALI/ARDS. However, in the search for a common early insult which occurs before the onset of clinical disease hypoxia seems to be an area of great potential.

Results of the work described above suggest that the alveolar macrophage may be a key target for the injurious stimulus that initiates ALI/ARDS. The lung is composed

of a number of different resident cell populations (including Type I and Type II alveolar epithelium, pulmonary endothelium and macrophages) which could singly, or in concert, trigger the cascade of inflammatory mediators that results in ALI/ARDS. However, the alveolar macrophage occupies a prime position to sense injurious stimuli at the air/fluid interface [71] and is able to respond to such stimuli by secreting a number of mediators which could trigger an inflammatory response (see table 1-2). Although well recognised that there is a massive pulmonary neutrophil influx in ARDS, it is less recognised that in both humans [72] and animal models [73;74], there is also an expansion of the macrophage pool. Furthermore, in humans there is evidence of activation of alveolar macrophages (specifically activation of transcription factors) early in the onset of ALI/ARDS [75].

Table 1-3 Mediators released by Alveolar Macrophages potentially involved in Acute Lung Injury

- Cytokines
  - Interleukins-1/6/8
  - Tumour Necrosis Factor
  - Interferon-α/γ
  - Colony stimulating Factors
  - Transforming Growth factor-β
  - Fibroblast Growth factor
  - Platelet Derived Growth Factor
  - Vascular Endothelial Growth Factor
- Enzymes
  - Lysosyme
  - Acid hylauronidases
  - Angiotensin Converting Enzyme
  - Elastases
  - Collagenases
  - Plasminogen activator
- Biologically active lipids
  - Cycloxygenase metabolites- Thromboxane A_2, Prostaglandins
- Oxygen metabolites
  - Hydrogen Peroxide, Superoxide, Hydroxyl radical
  - Nitric oxide
- Proteins
  - Antiproteases- plasminogen activator inhibitor-1
  - Complement components-C2, C4

In animal models of ALI, activation of the NF-κB transcription pathway in alveolar macrophages occurs before pulmonary damage[76] and depletion of the alveolar macrophage population results in striking attenuation of lung injury in a number of models[76-78]. To further support the importance of the alveolar macrophage it has been shown by adoptive transfer experiments that BAL cells from rats exposed to intratracheal LPS can induce ALI in naïve animals[79]. While some of this damage is undoubtedly due to transfer of neutrophils it is recognised that ARDS can occur in neutropenic patients[80] and that the resting non-inflamed lung has no resident alveolar neutrophils [71] suggesting that, while the neutrophil is the major effector cell in ALI [81], another cell is responsible for detecting and responding to the initial stimulus. Hypoxia has been shown to trigger the release of key mediators in ALI/ARDS such as IL-8 and TNFα [82;83] by alveolar macrophages.

The alveolar macrophage is also able to respond to other injurious stimuli which are seen in patients with ALI including hyperoxia [82;84-86], mechanical stretch[87] and haemorrhagic shock and sepsis [88].

The possible importance of the hypoxic sensing and control of the inflammatory response in ALI/ARDS was further enhanced by the identification of a novel key transcription factor Hypoxia inducible factor-1 (Hif-1) which seemed to play a key role in the development of inflammation [89].

1.2 Hypoxia inducible factor-1

1.2.1 Identification and description of Hypoxia inducible factor-1

The gene coding for the hormone erythropoietin (EPO) was cloned in 1985[90] and it was subsequently shown that EPO levels increase in response to hypoxia[91]. Furthermore this signal was not detected by the electron transport chain as cyanide did not reproduce the effect. Gregg Semenza's laboratory subsequently identified the transcription factor, Hypoxia-inducible factor 1 (Hif-1) as the key mediator of the transcriptional response to cellular hypoxia[92].

Hif-1 is a heterodimer composed of an α and β subunit (Figure 1-3)[92]. The 789 amino acid β subunit is constitutively expressed in all cells and is also known as aryl hydrocarbon nuclear receptor translocator (ARNT) [93]. Human Hif-1α is an 826 amino acid protein which is constitutively expressed in all nucleated cells. It is a member of the basic-helix-loop-helix (bHLH) PAS (Per-Arnt-Sim) family of transcription factors. The Human Hif-1α gene, *HIF-1A,* is encoded on chromosome 14 (14q21-24) [94] and continuous transcription is initiated by Sp1 binding sites in the 5' region. The murine *hif-1a* gene is encoded on chromosome 12 [94] and shares a 90% amino acid identity with the human equivalent.

Hif-1β is also a member of the bHLH PAS family and both Hif-1α and -1β contain 2 PAS domains (PAS A and B) in the amino terminal of the proteins. These 100-120 amino acid long domains form 5 stranded anti-parallel β sheets which are flanked by several α helices [95] and mediate heterodimer formation and DNA binding [96].

The remainder of the Hif-1α protein has also been characterised and has been shown to be composed of a number of subunits that are critical to its oxygen sensitive regulatory role. The COOH terminal of Hif-1α contains 2 transactivation domains, one NH2-terminal transactivation domain, (N-TAD), located at amino acids 531-575 and the other, COOH-terminal (C-TAD), which are essential for the transactivation activity of Hif-1. These 2 regions show 100% homology between mouse and human Hif-1α [97]. Between these two regions is the Inhibitory Domain (amino acids 576-785) and this has a 75% homology between human and mouse. Both N-TAD and C-TAD are able to initiate transcription of Hif-1 target genes however C-TAD also requires the binding of CBP/p300 for activity. There are two nuclear localisation signals which are localised to the amino terminal (amino acids 17-74) and carboxy terminal (amino acids 718-721) [96]. The region between amino acids 401 and 603 (i.e. overlapping with N-TAD but separate to C-TAD) is the Oxygen Dependent Degradation (ODD) domain [98] responsible for the unique oxygen mediated regulation of Hif-1.

The human HIF-1A gene has been characterised and is composed of 15 exons and 14 introns. Exon 2 codes for the bHLH sequence, exons 3-8 code for the 2 PAS domains while 9-15 code for the TAD/ODD domains. A number of isoforms of Hif-1α have been identified (see Lee et al [99]) but their exact functions remain uncertain. There are two other members of the bHLH-PAS superfamily Hif-2 and Hif-3. Hif-2 shares the same β subunit as Hif-1α. Hif-2α is also known as endothelial PAS [EPAS] protein, HIF-related factor [HRF], HIF-like factor [HLF] and member of the PAS superfamily 2 [MOP2][100]. Hif-2α shares a 48% homology with Hif-1α [94]. The expression of Hif-2 is more limited [101;102]. The differential roles for Hif-1 and Hif-2 are unclear. Hif-1α and 2α are not functionally redundant as knockout of either

is embryonically lethal. Hif-1α knockout mice die by day 11 [94;103] as a result of lack of blood vessel formation and neural fold defects. All glycolytic enzymes are Hif-1 regulated [104] while some genes are targets for both Hif-1 and-2 (e.g. Vascular Endothelial Growth Factor) . Hif-3α does not have a TAD and may be a negative regulator of hypoxic responses [105-107].

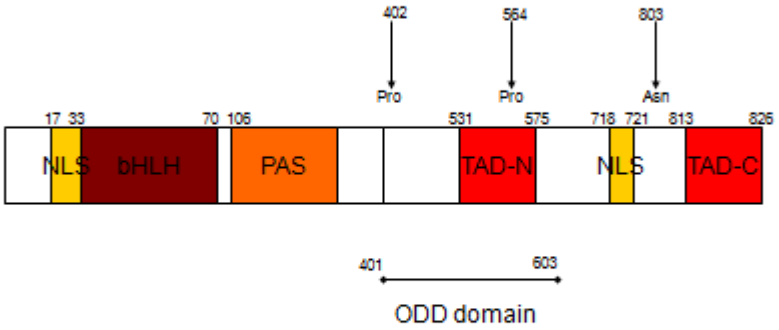


Figure 1-3 Structure of Hypoxia inducible factor-α

1.2.2 Hif-1 mechanism of action and target genes

Transcription factors are endogenous substances (usually a protein) that are effective in the stimulation /initiation of the genetic transcription process. Both Hif-1α and -1β are constitutively expressed in the cytoplasm. Following nuclear translocation and dimerisation they bind to target gene DNA via the Hypoxia Response Element (HRE; 5'-RCGTG-3') and initiate transcription. For full activity Hif-1α must bind CBP

(CREB binding protein)/p300 at the C-TAD [108]. This occurs via the Cysteine/Histidine-rich 1 (CH-1) domain of CBP/p300. The binding of CBP/p300 has 2 functions. First it acts as a link between the DNA binding protein and the transcription initiation complex containing RNA polymerase II. CBP/p300 also has histone acetyltransferase activity which allows chromatin remodelling required for transcription [109].

1-2% of human genes are regulated by hypoxia [110]. There are now over 70 genes that have been identified as Hif-1 regulated (Table 1-3). The central role of Hif-1 is to promote the cell's adaptation to hypoxia. This is done by increasing the cell's ability to synthesis ATP from glucose by glycolysis by increasing the enzymes controlling glucose uptake (GLUT-1) and glycolysis (LDH-A) [95]. Other target genes increase the delivery of oxygen to cells (EPO, transferrrin, VEGF).

The key control of Hif-1 function, both transcription initiation and termination of target genes, is by the cell's ambient oxygen level.

Table 1-4 Hif-1 Regulated Genes

- **Energy metabolism**
  - Glucose transporter-1
  - Hexokinase-2
  - 6-Phosphofructo-1-kinase
  - Glyceraldehyde-3-phosphate dehydrogenase
  - Adololase-A
  - Phosphoglycerate kinase-1
  - Lactate dehydrogenase-A
- **Hormonal regulation**
  - Erythropoietin
  - Leptin
- **Angiogenetic signalling**
  - Vascular endothelial growth factor-A
  - Endothelial-gland-derived vascular endothelial-growth factor
  - VEGF Receptor-1 (Flt-1)
  - Plasminogen activator inhibitor-1
- **Vasomotor regulation**
  - Endothelin-1
  - Adrenomedullin
  - Tyrosine hydroxylase
  - α_1-adrenergic receptor
  - Inducible nitric oxide synthase (Type II NOS)
  - Haem-oxygenase-1
  - Atrial natriuertic peptide
  - Endoglin
- **Growth and apoptosis**
  - Insulin-like growth-factor-binding-protein-1
  - Nip 3
  - Wilms tumour suppressor
  - Calcitonin-receptor-like receptor
  - Stromal derived factor-1 (CXCL12)
  - CXCR4
- **Transport**
  - Transferrin
  - Transferrin receptor
  - Ceruloplasmin
  - Multidrug-resistance P-glycoprotein
- **Transcriptional regulation**
  - CITED2 (p35srj)
  - ETS1
  - Differentiated Embryo-chondrocyte Expressed Gene-1 and -2
- **Inflammation**
  - Cycloxygenase-2
  - Adrenomedullin
  - Adenosine A2B receptor
- **Nucleotide metabolism**
  - Adenylate kinase 3
  - Ecto-5’-nucleotidase
- **pH regulation**
  - Carbonic anhydrase-9

1.2.3 Regulation of Hif-1α protein levels and activity by oxygen

The control of Hif-1 protein levels and activity is controlled by the cell's ambient oxygen level (summarised in figure 1.4). The control is focussed on the Hif-1α subunit. If Hif-1α is over expressed in a cell the activity at reporter HRE increases confirming that Hif-1β is present in excess and is not the limiting factor [97].

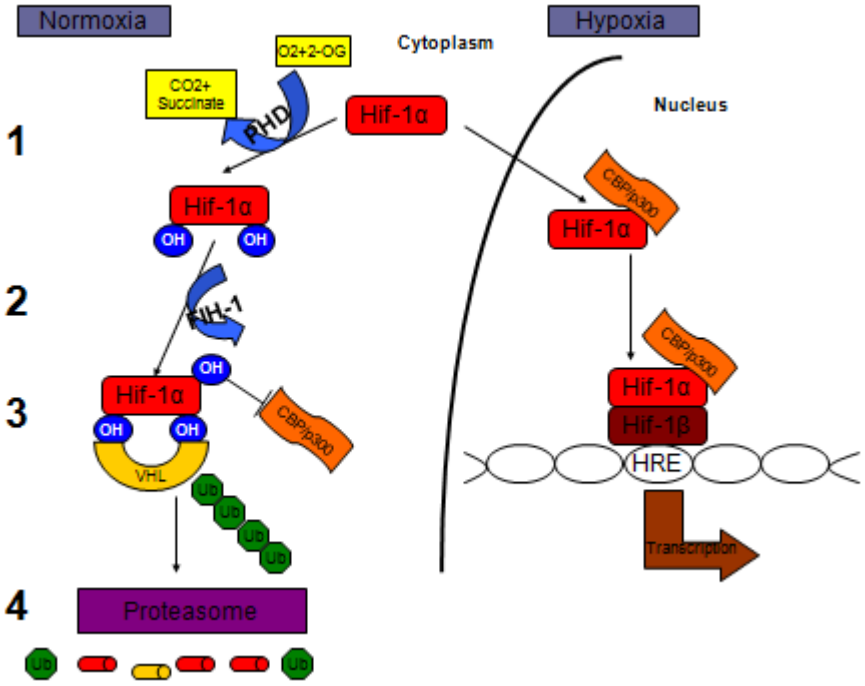


Figure 1-4 The oxygen dependent regulation of Hif-1α.

The oxygen dependent degradation of Hif-1α can be viewed in 4 stages.

Stage 1: In the presence of oxygen PHDs hydroxylate Proline residues 402 and 564

Stage 2: FIH-1 can also hydroxylate Aspargine 803
Stage 3: The hydroxylated Proline residues allows vHL to bind to Hif-1 while the interaction between Hif-1 and the transcription cofactors CBP/p300 is inhibited by the Asn hydroxylation
Stage 4: The binding of the vHL/ubiquitin complex targets Hif for destruction by the 26S proteasome complex.
O2 oxygen; 2-OG 2-oxoglutarate; PHD prolyl hydroxylase; OH hydroxyl group: FIH-1 Factor Inhibiting Hif-1: VHL von Hippel Lindau protein: Ub Ubiquitin: CBP/p300 CREB binding protein.

The half life of de novo synthesised Hif-1α in vitro is about 5 minutes [111] and is dependent on the presence of the ODD [98]. In the presence of oxygen Hif-1α is targeted for degradation by the product of the von-Hippel Lindau (pVHL) tumour suppressor gene [112;113]. pVHL binding directs the formation of the VCB-Cul2 E3 ligase composed of elongin C, elongin B, cullin-2 and Rbx1 [114]. This multiprotein E3 ligase causes the polyubiquitination of the Hif-1α subunit and targets it for degradation via the 26S proteasome pathway [95;111;115]. The key discovery in understanding the oxygen regulated control of Hif-1α protein levels was the discovery that the mechanism for pVHL recognition of the -1α subunit required the hydroxylation of 2 proline residues in the ODD. In the presence of oxygen Pro 402 and 564 are hydroxylated by a family of enzymes the Prolyl Hyrdoxylases (PHD) [116;117]. PHDs belong to the 2-oxoglutarate dioxygenase family which are non-haem Fe2+ dependent enzymes [118]. There are 3 isoforms (PHD 1-3) which were isolated on the basis of their homology to the elg9 hydroxylase enzyme of the nematode *Caenorhabditis elegans* [119]. The major functionally active isoform of PHD is PHD2 [120]. The physiological function of the remaining isoforms is unclear. To function PHD require the presence of oxygen, Fe2+ and 2-oxoglutarate. The proline hydroxylation reaction results in one oxygen atom being transferred to the Pro residue while the other reacts with 2-oxoglutarate to form succinate and carbon dioxide. Ascorbic acid catalyses the reaction by maintaining iron in the ferrous state (Fe2+) [119;121].

The nature of the oxygen sensor remains an area of debate but a unifying model can be conceived which allows the experimental results of differing groups of investigators to be reconciled. (The regulation is summarised in Figure 1-3)

Conceptually the simplest view is to consider the situation in anoxia. Here in the absolute absence of oxygen Hif-1α is stabilised as a result of lack of substrate for the PHDs. The measured Km [O2] for recombinant PHD is in the range of 230-250μM [122] (the Km [O2] for complex IV of the mitochondrial respiratory chain is <1μM). These values are above the physiological (and therefore pathophysiological) levels of cellular oxygen and hence these enzymes are acting under non-equilibrium conditions and can act as the oxygen sensor. This model suggests that in hypoxia (as opposed to anoxia) as oxygen availability decreases (and the majority of that available is consumed by the respiratory chain) there is a corresponding decrease in the activity of the PHD and hence an increase in cellular Hif-1α levels.

However there is a dispute that the oxygen sensor is the PHD. It has been shown in Hep3B cells that in hypoxia (as opposed to anoxia) that the presence of functioning mitochondria is required to allow Hif-1α stabilisation [123]. Rho zero cells (mitochondrial deficient cells) while able to stabilise Hif-1α in anoxia are unable to stabilise Hif-1α in hypoxia. Equally inhibitors of mitochondrial function are able to stop the hypoxia mediated Hif-1α stabilisation. Furthermore in the absence of mitochondria the stabilisation of the α subunit by iron chelators is maintained. Chandel et al [123] have suggested that the key to understanding the role of the mitochondria as the oxygen sensor is to propose that in hypoxia there is increased generation of reactive oxygen species (ROS) at complex III of the electron transport chain. In the presence of increased ROS there is altered cellular Fenton chemistry with an increase in ferric (Fe^{3+}) iron which inhibits the action of the PHDs which require ferrous (Fe^{2+}) iron.

The two views of the PHD or the mitochondria as the oxygen sensor do not have to oppose and it would seem that an increase in the proportion of oxygen being utilised by the electron transport chain, reducing the availability of oxygen to the PHDs plus an alteration in the redox state of the PHD cofactors together can act as a rapidly responsive system to changes in cellular oxygen.

The activity of stabilised Hif-1α is further regulated by the cellular oxygen level. For full activation the C-TAD requires the binding of CBP/p300. This is controlled by the activity of another hydroxylase- Factor inhibiting Hif-1 (FIH-1). In the presence of oxygen FIH-1 hydroxylates Asn 803 which prevents the interaction with CBP/p300 [124;125]. Furthermore FIH-1 acts as a repressor of Hif-1α activity by recruiting Histone deacetyltransferases resulting in an unfavourable chromatin structure for transcription [126].

Finally prolonged hypoxia may result in altered Hif-1α transcription. This has recently been shown in A549 cells where prolonged (>12 hours) resulted in reduced levels of Hif-1α mRNA [101]. This was proposed to occur through an increase in Hif-1α antisense mRNA [127] which is increased in hypoxia.

1.2.4 Regulation of Hif-1α by non-hypoxic pathways

There has recently been increasing evidence that Hif-1 is also controlled through non-oxygen mediated pathways, mediated via more classical signalling pathways (see figure 1-4). A range of signalling molecules including insulin, platelet derived growth factor, epidermal growth factor, fibroblast growth factor-2, TNFα and thrombin (see [128] have been shown to increase either Hif-1α mRNA or protein levels. These molecules signal via two pathways- the Mitogen Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-kinase/Akt (PI3K) pathways. Using Chinese hamster fibroblasts (CCL39) Richard et al [129] have shown that Hif-1α is phosphorylated by p42/44 and that the phosphorylated Hif-1α has greater transcriptional activity. Recently Sang et al [130] have also shown that in the human cervical carcinoma HeLa cell line CBP/p300 can be phosphorylated by MAPK and that this increases the transcription signal. Also Hif-1β can preferentially bind the phosphorylated form of Hif-1α [131] (in a breast carcinoma cell line MCF-7).

Others have shown that the PI3K pathway can result in increased Hif-1α protein levels in normoxia [132-135] (in a range of cells including porcine proximal renal tubular LLC-PK1 cells[132]; the human liver carcinoma lineHepG2 [133]; the glioblastoma cell line U373 [134] and the mouse embryonic fibroblast cell line NIH3T3 [135] (see diagram from Bilton& Booker [128]). These signals generate an increase in protein levels which is thought to be enough to overwhelm the PHD activity and result in a small increase (though less than seen in hypoxia) in active α subunit.

Other transcription factors also affect Hif-1 dependent pathways. p53 can decrease the activity of Hif-1α via several pathways. Blagosklonny et al [136] have shown that p53 can inhibit CBP/p300 interaction in human carcinoma cell lines (SKBr3, MCF7 human breast cancer cell lines and PC3M a highly metastatic variant of the prostate cancer cell line, PC3) as both p53 and Hif-1α bind to the CH1 rich region. Also in a colorectal carcinoma line (HCT116) p53 increases the levels of mdm-2 an alternative E3 ubiquitin ligase which can target Hif-1 for proteosomal destruction [137].

These discoveries suggest that there exists a range of controlling pathways which are able to fine tune the level/activity of Hif-1 in concert with the cell's ambient oxygen level.

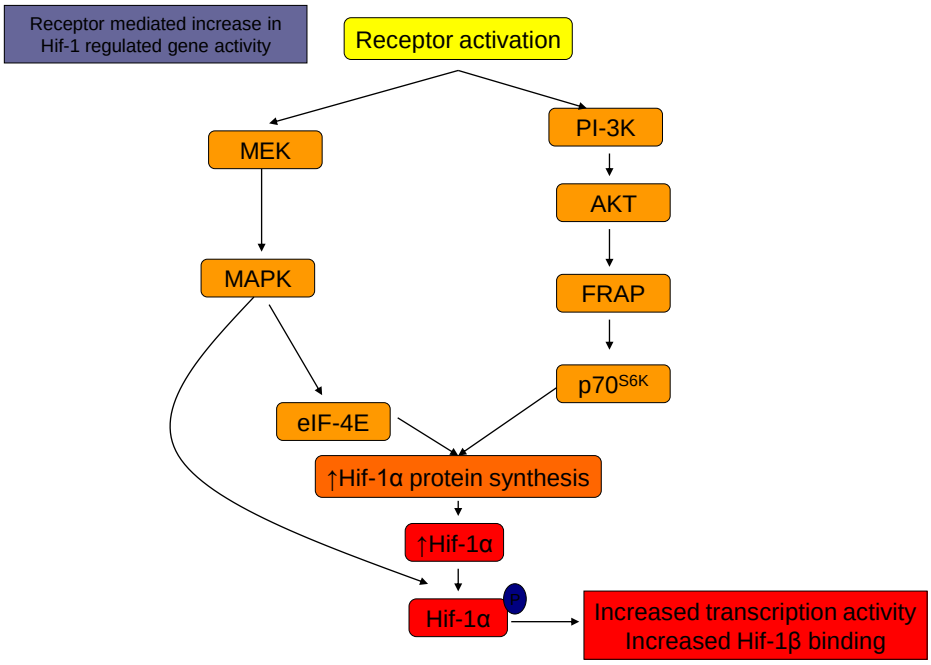


Figure 1-5 Non-hypoxic regulation of Hif-1α

Receptor activation leads to signalling cascades via the MAPK and PI3K pathways which lead to increased Hif-1α protein synthesis and activity.
MEK MAPK kinase; MAPK Mitogen Activated Protein Kinase; PI-3K Phosphatidyl Inositol 3 kinase; AKT serine/threonine protein kinase Akt ; FRAP FKBP12-rapamycin complex-associated protein; p70^{S6K} ribosomal protein S6 kinase, 70kDa, polypeptide 1; eIF-4E eukaryotic translation initiation factor 4E

1.3 Role of Hif-1 in inflammatory disease.

Pro-inflammatory signal such as IL-1β and TNFα have been shown in vitro to increase Hif-1α mediated signalling [138]. It has also been shown in primary inflammatory cells that Hif-1α protein is increased in a model of tissue injury [139].

A recent key paper [89] has suggested that Hif-1α may have a central role in controlling the myeloid cell mediated inflammatory response. Using a myeloid cell conditional knockout mouse (exon 2 targeted and therefore deficient in the bHLH sequence [140]) Cramer et al [89] were able to shown reduced myeloid cell function both in vitro and in several murine models of inflammatory injury. Kong et al [141] have also shown that the migration of inflammatory cells is also regulated in a Hif-1α dependent manner. Finally in macrophages Blouin et al [142] have shown that endotoxin, a key possible trigger of ALI, can increase both Hif-1α mRNA and protein via the PI3K pathway.

On the basis of this work this thesis aimed to undertake a study of the role of Hif-1α in acute lung injury, specifically studying its role in the alveolar macrophage.

1.4 Aims and hypothesis

This thesis was undertaken to test the hypothesis that hypoxic and non-hypoxic signals via the Hif-1 transcription apparatus controlled the alveolar macrophage response to stimuli associated with the development of acute lung injury.

In order to test this, the aims of this work were to:

1. Develop a model of acute lung injury in mice.

2. Study the effect of Hif-1α depletion in the myeloid cells of mice using the model.

3. Study the effects of manipulating Hif-1α *in vitro* both via hypoxic culture and pharmacologically in an alveolar macrophage cell line in response to a well recognised inflammatory stimulus (endotoxin).

4. Determine the feasibility and effects of manipulating Hif-1 in vivo in the model of lung injury.

Chapter 2

Materials and Methods

2.1 Tissue Culture

2.1.1 General cell culture

Media

Unless otherwise specified medium was RPMI 1640 (PAA Laboratories, Yeovil, UK) supplemented with10% foetal calf serum (FCS) (GIBCO BRL, Life technologies Paisley UK), 2mM L-glutamine (PAA), 50μM β-mercaptoethanol (PAA) and 100U/ml penicillin and 100μg/ml streptomycin (PAA).

PBS for cell culture was purchased from PAA.

Plasticware

All tissue culture flasks and plates were supplied by Corning Life Sciences.

Cell line

The alveolar macrophage cell line MH-S (ATCC CRL-2019) was derived from Simian Virus 40 transformed alveolar macrophages obtained from Balb/cJ mice [143] was obtained from the ATCC. They have been shown to have functional similarity to primary alveolar macrophages [143;144]. MH-S cells express well characterised murine macrophage surface antigens including CD11b, CDw32 (FcγII Receptor),

F4/80 and class II MHC. As would be expected they do not express CD4 or CD8. MH-S cells secrete IL-1 and nitric oxide in response to endotoxin.

Cells were cultured at 37^0C in a 5% CO_2 atmosphere routinely.

Cells were passaged every 2-4 days when subconfluent. After washing excess media with PBS cells were incubated for 10 minutes in Trypsin/EDTA (0.5mg/ml and 0.22mg/ml respectively) solution (PAA) at 37^0C. The tryspin was then inhibited by addition of an equal volume of complete medium and the cell suspension then centrifuged at 300xG for 5 minutes. The cell pellet was then resuspended and the cell number counted using a haemocytometer. Culture flasks were then seeded at a density of $1x10^5$cells/ml in an appropriate volume of complete medium.

For experiments cells were split as described and then plated into appropriate sized tissue culture plates. Generally cells were plated in triplicate for each experimental condition.

2.2 Cell assays

2.2.1 Lactate Dehydrogenase Assay

As a measure of cell death the release of Lactate Dehydrogenase (LDH) was measured in the supernatant in some experiments. This was done using the *in vitro* Toxicology assay TOX-7 (Sigma Aldrich, Dorset, England). To allow calculation of percent cell death triplicate wells were treated with the supplied lysis solution which reproducibly results in complete death of all the cultured cells.

2.2.2 Nitrite assay

The production of Nitric oxide (NO) was measured using the Griess assay which measures the level of Nitrite (NO^-_2) in medium, one of the stable breakdown products of NO. This was done using the colourimetric Griess Reagent system (Promega Corporation, Madison, WI, USA). The sensitivity of the assay is 2.5μM nitrite. Assays were performed in 96 well plates and read at 520nM using a plate reader and Gen 5 software.

2.2.3 Enzyme-linked Immunosorbent Assays

Cytokine levels in cell supernatant and bronchoalveolar lavage fluid were measured in accordance with manufacturers' instructions in 96 well plates. Murine TNF-α kits were supplied by Insight Biotechnology (Wembley,UK). Murine CXCL 12 and murine MIP-2 were measured with kits supplied by R+D (R+D Systems, Abingdon, UK).

An example of a standard curve generated using the TNF assay is shown in figure 2.1.

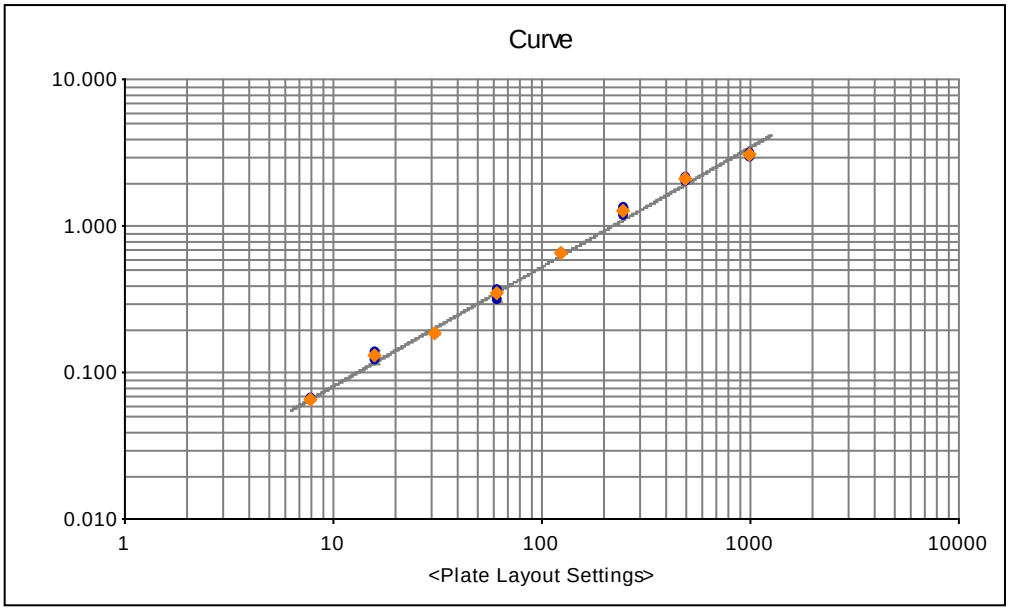


Figure 2-1. Standard curve generated using the TNF ELISA. Duplicate wells were run using serial dilutions from 1000 to 8pg/ml.

2.2.4 Cytometric Bead array assay

In some experiments cytokines were measured using the BD Cytometric Bead Array system (BD Biosciences, Oxford, UK) which allows the simultaneous measurement of 6 murine specific cytokines (IL-6; IL-10; IL-12; MCP-1, IFN-γ and TNF) in the same sample. Manufacturers' instructions were followed other than reagent volumes were halved in order to increase the number of tests per kit as is standard practice in this laboratory. Samples were processed on a BD FACSCalibur cytometer with BD CellQuest software.

2.3 Total protein extraction

To collect total cell protein for western blot analysis cells were cultured in 100mm tissue culture treated culture dishes. Following exposure to the experimental condition(s) total cell protein was collected as follows.

Dishes were transferred on ice and the conditioned medium was decanted off and the cells were washed twice with ice cold PBS. Cells were then lifted by scrapping in 0.5l of cold PBS and pelleted by centrifugation at 6000rpm for 2 minutes at 4^{o}C. Cells were then lysed by the addition of an appropriate volume of RIPA buffer (Pierce, Thermo Fisher Scientific, Cramlington, UK) containing Halt Protease inhibitor cocktail (Pierce). The lysate was then agitated for 15 minutes on ice and then the cellular protein was collected by centrifugation at 13000 rpm for 15 minutes at 4^{o}C to pellet cellular debris. The supernatants were aliquoted and then stored at -70^{o}C until used.

2.4 Nuclear Protein extraction

In order to perform Western blots to study the levels of Hif-1α it was necessary to collect separate nuclear and cytoplasmic fractions of cellular protein. Cells were again cultured in 100mm tissue culture treated culture dishes. At the end of the experimental period the cells were transported on ice and then quickly washed twice in ice cold PBS. Cells were then collected by scrapping and collected in cold PBS. Following centrifugation at 6000rpm for 2 minutes at 4^{o}C the cell pellet was collected and the cells were lysed using NE-PER nuclear and cytoplasmic extraction kit (Pierce). In

order to maximise the concentration of nuclear protein obtained the recommended volumes of reagents CER I and CER II were used but the volume of NER was halved. Protease inhibitor cocktail was added at both the cytoplasmic and nuclear extraction steps. The cytoplasmic and nuclear extracts were both stored at -70^{o}C until needed.

2.5 Protein Assay

To quantify the amounts of nuclear, cytoplasmic or total protein obtained by the above procedures the protein concentration was measured using the BCA protein assay (Pierce) which is not affected by the components of the lysis buffers. Manufacturer's instructions were followed for the microplate procedure which depends on the development of a purple colour following the reduction of copper by alkaline media and its subsequent chelation by bichinoninic acid. Assays were performed in a 96 well plate and read at 562nm by a plate reader and Gen 5 software. Protein standards were run in triplicate and samples in duplicate in order to conserve samples.

2.6 Western blot

2.6.1 General Protocol

Protein extract was mixed with an appropriate volume of NuPAGE (x10) reducing agent and Loading buffer (x4) (Invitrogen) and heated for 10 minutes at 70 ^{o}C.

The protein was then resolved on pre-cast Novex Bis-Tris Gels (Invitrogen) by electrophoresis at 200V for 35 minutes. Protein was then transferred to PVDF (Amersham). Membranes were blocked with 5% milk (Marvel) in Tris buffered saline (TBS) for 30 minutes at room temperature. Primary antibody incubation was performed overnight at 4 ^{o}C. The membrane was then washes 3 times in TBS/0.1% Tween (TBS-T). A species appropriate polyclonal HRP conjugated antibody was then incubated for 1 hour at room temperature. Following three further washes with TBS-T signal was detected by chemiluminescence using SuperSignal West Femto (Pierce). Images were obtained using a Bio-Rad Versa Doc 4000 imaging system. Images were stored on a PC using the supplied Quantity one software.

2.6.2 Antibodies used in Western blots

Primary antibodies

The following antibodies were used to detect proteins of interest.

Hif-1α polyclonal antibody (Cayman Chemicals). A polyclonal rabbit IgG raised against the C-terminal amino acids 809-826 of the Hif-1α protein. A final concentration of 5ng/ml was used.

Hif-1α monoclonal antibody (BD Transduction Laboratories, BD Biosciences, Oxford) A monoclonal mouse IgG_1 directed against the C-terminal amino acids 610-727 of the Hif-1α protein. This antibody failed to detect murine Hif-1α by Western blot or immunohistochemistry.

Hif-1α monoclonal antibody (Abcam). A mouse monoclonal IgG_{2b} directed against amino acids 432-528 of Human HIF1 alpha. This antibody was able to faintly detect murine Hif-1α protein on Western blot (see in vivo chapter).

nNOS rabbit polyclonal antibody. (BD Biosciences). This antibody was raised against the C-terminal amino acids 1095-1289 of human nNOS. A final concentration of 0.5μg/ml was used.

iNOS Mouse IgG1 monoclonal antibody (BD Transduction Lab). This antibody generated against the C terminal amino acids 961-1144 of mouse iNOS was used at a final concentration of 0.25ng/ml.

eNOS rabbit polyclonal antibody (Abcam) This antibody has been raised against amino acids 1179-1194 of human eNOS. This was used at a final concentration of 0.2ng/ml.

Cre rabbit polyclonal antibody (Novus Biologicals Littleton, CO, USA) This antibody was raised against the 35kDa bacteriophage P1 Cre recombinase. It was used at a dilution of 1:10000.

Heme-oxygenase-1 antibody (Stressgen, Ann Arbor, Michigan, USA). Raised against native rat liver HO-1 protein this antibody detects a 32kDa protein. It was used at a final dilution of 1:5000

Secondary antibodies

Species appropriate Horse Radish Peroxidase (HRP) labelled antibodies were obtained from Dako (Dako Cytomation, CA, USA). These were used at a final dilution of 1:10000 as per the instructions in the Supersignal West Femto system.

2.3 Hypoxic and hyperoxic cell culture conditions

Variable oxygen conditions were achieved in a humidified variable aerobic chamber (Oxycycler Model C42, Biospherix Limited, Redfield, New York). This system is controlled by a PC running Watview Runtime Version 2.5.63 and rapidly achieves the desired ambient oxygen condition by controlling the inflow mixture of oxygen and nitrogen. All cell culture was performed in the presence of 5% CO_2. The chamber was calibrated by the supplier's engineer using precision generated calibration gas to ensure accurate results during regular servicing.

2.4 Animal Techniques

All animals used in this thesis were housed in the Animal Facilities of the Biomedical Research Resource Unit of the University of Edinburgh. Initially the facility in the Hugh Robson building was used but the animals were moved to the new facility in the Chancellor's Building, Little France. These facilities are specific pathogen free. All animals were maintained on a 12 hour day/night cycle and had unrestricted access to food and water.

All procedures were performed in accordance with Home Office Regulations and had Local Ethical Committee approval. As the models investigated in this thesis were deemed to be severe insults there was frequent oversight by local and Home Office veterinary surgeons.

2.4.1 Purchased animals

FVB male mice were purchased from Harlan (Huntington, England). Mice were purchase aged 6-8 weeks and allowed to acclimatise for at least one week before use in an experiment. These mice were housed singly to prevent fighting.

The FVB strain is derived from White Swiss mice [145] and has been found to be useful in transgenic research due to the relatively large size of its pronucleus [146]. This has lead to its use in a number of studies characterising the response to inflammatory insults in the lung [147;148], systemically [149;150] and in other organs [151;152]. FVB mice have physiological reactions to insults such as endotoxin [149;153] and hypoxia [154] similar to those seen in humans. Furthermore, FVB

strongly express CD14 on macrophages but much less intensely on other tissues [149] and as this thesis is concerned with the macrophage response to insults this may be a benefit.

2.4.2 On-site bred animals

The Myeloid cell specific knockout animals used in this thesis were a kind gift from Professor R. Johnson of the University of California, San Diego, USA. They were rederived by the staff of the BRR facility and bred in a pathogen free environment. Genotyping was performed from ear clips obtained by the BRR staff. Animals were designated as Cre negative (i.e. wild type) or positive (i.e. knockout) by real time PCR using in house designed probes by the BRR facility. In experiments, where possible, cre positive and negative litter mates were used.

2.4.3 General Anaesthesia

In this thesis two methods of inducing general anaesthesia were used.

Initially animals were anaesthetised by inhalation of the volatile anaesthetic Isoflurane. This was done using oxygen as the carrier gas and results in the rapid induction of surgical anaesthesia. This technique results in the rapid recovery of the animals once removed from the volatile agent and this technique was used during the haemorrhagic shock model.

The other method of anaesthesia was by intraperitoneal injection of Avertin (Tribromoethanol anaesthetic). This was used from a pre-prepared frozen stock of ; 2.5g 2,2,2-tribromoethanol (Sigma-Aldrich), 5ml 2 methyl-2-butanol tertiary amyl alcohol; (Sigma-Aldrich); made up to 200ml with dH_2O. This results in a slower onset of anaesthesia but the standard dose results in an approximate 30 minute long period of surgical anaesthesia with a further period of more prolonged recovery.

2.4.4 Tissue collection

At the end of the experimental period animals were sacrificed by an approved method. In animals undergoing bronchoalveolar lavage euthanasia was performed by the administration of a lethal dose of pentobarbitone and following the loss of reaction to painful stimuli the abdominal cavity was opened and the animal was exsanguinated by aorto-caval transaction.

Tissue was collected for further study by the following methods

Bronchoalveolar lavage

Once the animal was dead the thoracic cavity was opened to expose the contents. The trachea was exposed and cannulated with a section of polyvinyl chloride tubing (0.67mm internal diameter). 0.8ml of cold PBS was then slowly instilled three times to lavage the lungs. The first lavage was kept separate from lavages 2 and 3 to allow the supernatant to be kept for subsequent cytokine analysis. From lavages 2 and 3 only the cells were retained for analysis. Lavages were kept on ice until further processing in the laboratory.

In order to minimise the risk of contaminating the BAL contents the animals were exsanguinated prior to BAL in order to achieve a bloodless field during the neck dissection. It was decided that flushing the vasculature was not appropriate prior to BAL in order to minimise pulmonary tissue trauma that can occur during the cannulation of the pulmonary artery and incision of the left atrium. Furthermore the release of intravascular fluid could contaminate the collected BAL fluid. In addition the flushing of the vasculature could dislodge intravascular features of ALI (for

example migrating neutrophils, microvascular thrombi) and it was felt that the ability to see this features would be of benefit.

Lung fixation

Following BAL the heart, lungs and other mediastinal contents were removed en bloc. 1 ml of fixative (10% neutral buffered formalin [Sigma Aldrich] or Methacarn [60% methanol 30% chloroform 10% glacial acetic]) was instilled and then the trachea was tied. The lung block was then submerged in fixative for 24 hours before processing and embedded in paraffin wax. Blocks were stored at lab temperature out of direct light. Sections for immunohistochemistry were cut at 3μm thickness on a microtome and floated at 42^{o}C prior to mounting on superfrost slides (BDH, Poole, UK). Slides were incubated overnight at 37^{o}C and then stored at room temperature away from direct light.

2.4.5 Bronchoalveolar lavage processing

Both samples of lavage 1 and the combined sample of lavage 2 and 3 were centrifuged for 2 minutes at 500x G at 4^{o}C. The supernatant from lavage 1 was decanted and kept for further analysis. The supernatant from lavages 2 and 3 was discarded.

The 2 cell pellets were resuspended in a total of 500μl of PBS. The total cell count was then determined using the NucleoCounter (Chemometec, Sartorius, Surrey,

England) automatic mammalian cell counter system following the manufacturer's instructions.

A sample of the cell suspension containing approximately $2x10^5$ cells was then used to prepare slides (Superfrost) by centrifugation at 300rpm for 3 minutes (Cytospin 2, Shandon Instruments Pittsburgh, USA). After air drying the slides were stained using Diff Quik (Dade Behring Dudingen, Switzerland) again following the manufacturer's recommendations. BAL cell differential counts were then performed using standard morphological criteria under a light microscope at 40x magnification. A total of at least 200 cells were counted per slide.

Supernatant analysis

As a measure of alveolar capillary damage the lavage fluid protein concentration was determined using the BCA protein assay as described above. Samples were run in duplicate. BAL cytokine levels were measured using ELISAs as described above.

2.4.6 Lung Myeloperoxidase assay

As a measure of pulmonary neutrophil infiltration the level of myeloperoxidase (MPO) activity was measured in lung homogenate based on the method of Suzuki et al [155].

The right lung of some animals was snap frozen in liquid nitrogen and stored at -80^{o}C until required. After thawing the lung was added to 1ml of 100mM Na2HPO4 pH6 buffer and homogenised for 20 seconds. The homogenate was then centrifuged at 10000 rpm for 15 minutes at 4 ^{o}C and the supernatant discarded. The pellet was resuspended in 300µl of Na2HPO4 and 0.5% Hexadecyltrimethylammonium bromide and homogenised on ice. The sample was then sonicated for 20 seconds and then frozen/thawed 3 times. Following a final centrifugation step at 10000rpm for 10 minutes at 4 ^{o}C the supernatant was stored at -80 ^{o}C until used.

The MPO assay was performed by measuring the colour change caused by the reaction of 10µl of supernatant with 140µl of TMB superfast substrate (Sigma). The colour change at 655nm was recorded over a 3 minute time period. This was performed in a Cobas Fara II analyser (Roche).

2.4.7 Wet/Dry lung weight

As a measure of pulmonary oedema the wet/dry weight of the lungs of some animals was recorded [156]. Briefly the left lung of the animal was removed and blotted dry and then weighed. It was then placed in an oven at 65 ^{o}C for 2 days. The dry weight was then recorded and the difference (wet/dry weight) was determined.

2.4.8 Evans blue dye measurement for determination of pulmonary vascular permeability

Evans blue dye has a high affinity for albumin and binds stably to it seconds after plasma injection. The measurement of dye deposition in organs can therefore be used as a measure of vascular permeability in animal models[156].

Animals were injected intravenously with 20mg/kg of Evans blue (Sigma) dye 30 minutes prior to sacrifice. After sacrifice the pulmonary vasculature was flushed with saline until the fluid leaving the left atrium was clear. The lung was then homogenised in 1 ml of PBS to which 2ml of formamide was then added. The mixture was incubated overnight at 60 ^{o}C. Following this the sample was centrifuged at 2000xG for 30 minutes to remove cell debris. Evans blue in the lung tissue was quantitated by dual-wavelength spectrophotometric analysis at 620 and 740 nm (Sue et al 2004). This method corrects the specimen's absorbance at 620 nm for the absorbance of contaminating heme pigments, using the following formula: corrected absorbance at 620 nm = actual absorbance at 620 nm – (1.426 (absorbance at 740) + 0.03).

2.4.9 Immunohistochemistry

Protocols varied with antibody utilised. The common core protocol is described below and variations from this are described under specific antibody headings.

General Protocol

Methacarn fixed paraffin embedded sections were prepared for immunohistochemistry by dewaxing in xylene (Genta Medical, York, UK), where slides were submerged for 2x5minutes, followed by 1 minute submersions in 100%, 70% & 50% methylated spirit (Genta Medical) for serial rehydration to water. From here slides underwent antigen retrieval with antigen unmasking solution (Vector, CA, USA) where 5mls is added to 500mls of water in a plastic container, slides are then submerged and microwaved on full power for 3x5 minutes. Slides were then cooled for 20 minutes in flowing tap water. This was then followed by a peroxidase blocking step, where slides were incubated in a 2% H_2O_2 solution (Sigma-Aldrich) for 15 minutes, then washed in PBS for 2 minutes.

Slides were then loaded into Sequenza coverplate clips (Thermo Shandon, Cheshire, UK), which allow use of small 125µl volumes to cover sections through utilisation of capillary action across the surface of the slide. Reagent deliveries to these clips were separated by two to three full PBS washes.

Once the Sequenza was loaded, slides were biotin and avertin blocked using a Vector kit. Three drops of each (approx 125µl) were applied for 15 minute room temperature incubations, separated by PBS wash steps. This was then followed by the specific primary antibody. For rabbit polyclonal antibodies the Dako EnVision system (Dako

Cytomation, CA, USA) was then used to detect antibody binding using 3 drops of the labelled polymer. After 30 minutes incubation and 3 washes with PBS this was followed by use of the diaminobenzidine (DAB) chromogen development systems (Dako Cytomation, CA, USA), where 1 drop of concentrate was added per ml of supplied buffer and administered to sequenza for 5 minutes at room temperature. Slides were then counter stained in haematoxylin (Shandon/Thermo) for 5 seconds (to stain the nucleus) and rinsed in tap water for 5 minutes. This was followed by submergence in Scot's tap water (Sodium bicarbonate 17.5g, magnesium sulphate 100g (Sigma-Aldrich), five litres of water) for 1 minute. Slides were then rinsed in tap water and dehydrated through three alcohols and three xylenes, 1 minute each, prior to cover slip addition with DPX mountant.

Nitrotyrosine IHC

Anti-nitrotyrosine rabbit polyclonal antibody was supplied by Upstate cell signalling solutions (Lake Placid, NY, USA). This antibody has been raised against nitrated Keyhole Limpet Hemocyanin. For IHC protocols the antibody was used at a final concentration of 5µg/ml. Incubation was performed at 4^{o}C overnight.

To confirm specificity of the staining positive control slides were generated by incubating uninjured lung sections with peroxynitrite (catalog # 20-107). After the tissue section was deparaffinised, the slide was covered with a drop of PBS, pH 7.4 into which 10 microliters of peroxynitrite solution was mixed. Negative controls were prepared by omission of the primary antibody. To further confirm specificity of staining the primary antibody was blocked by incubation with a 10mM nitrotyrosine solution for 1 hour at room temperature.

Cre IHC

Rabbit polyclonal anti Cre (EMD Chemicals) was used at a final concentration of 5μg/ml. This antibody was raised against the 35kDa bacteriophage P1 Cre recombinase. Primary antibody was incubated for 1 hour.

Hif-1α IHC

This was performed using a rabbit polyclonal antibody (Cayman Chemicals, Ann Arbor, Michigan, USA) raised against the C terminal amino acids 809-826 of Hif-1α. It cross reacts with mouse and human protein. Antibody was used at a final concentration of 0.5μg/ml. To allow detection of this protein a modified protocol was followed. Antigen retrieval was performed using Borg Decloaker (BioCare Medical Concord, Califonia, USA) in a microwave pressure cooker. After overnight incubation with the primary antibody a biotinylated goat anti-rabbit antibody (Dako) was incubated for 40 minutes at a final concentration of 6μg/ml. Bound secondary was then visualised by incubation with streptavidin horseradish peroxidase and then DAB.

Pimonidazole IHC

This was done using the Hypoxyprobe-1 Plus system for the detection of tissue hypoxia (Chemicon International, Temecula, California, USA). This system uses the ability of a nitroimidazole compound, pimonidazole, to bind irreversibly to hypoxic proteins. Animals were injected with 30mg/kg of pimonidazole intraperitoneally 30 minutes before an experimental procedure.

Sections for immunohistochemistry was prepared as described above. Following the manufacturer's protocol pimonidazole binding was then detected by incubation with a FITC-labelled mouse anti-pimonidazole antibody at a concentration of 50μg/ml. A supplied HRP conjugated anti-FITC antibody was then used as secondary antibody at 50μg/ml final concentration.

2.5 Alveolar macrophage culture

BAL was performed as previously described. However, in order to maximise AM recovery 5 separate washes of 0.8ml of saline were used and lungs were gently massaged with blunt forceps. Isolates were pooled and kept on ice at all times to reduce cellular activity and macrophage loss through adherence to the bijou. The expected yield was1-2x10^5 cells per animal.

AM extracts were centrifuged at 300xg for 10minutes at 4^{o}C and the resulting pellet re-suspended in RPMI plus 10% FCS, 2mM L-glutamine, 50μM β-mercaptoethanol and 100U/ml penicillin and 100μg/ml streptomycin . Cells were plated with 2x10^5cells/well in a final volume of 1 ml medium.

Plated AM were left for 1-2 hours to adhere then the original medium was removed including contaminating red blood cells and replaced with fresh medium.

2.6 Bone Marrow Derived Macrophages

Bone Marrow derived macrophages (BMDM) were derived by standard techniques used in the CIR. Briefly mice were sacrificed by a schedule 1 approved method. The animal was then bathed in 70% ethanol, de-trousered and the femurs were removed by dissection. Care was taken throughout to avoid damage to the bone. The bone was then placed in cold PBS plus Pen/Strep and stored on ice until returned to the laboratory. Under sterile conditions the bones were then washed with 70% ethanol and remaining muscle was removed from the bone. The bone was then cut through both epiphyseal plates and using a 25G needle the marrow contents were flushed into a universal pot. Medium composed of Gibco's DMEM/F12 Glutmax, with 10% FCS, and Penicillin 100U/ml and Streptomycin 100μg/ml and 20% L929 medium (see below) was used to flush the marrow content. The cells were then resuspended by pipetting in the medium. The cells from one animal were then cultured in a 60ml Teflon pot for 7 days in a total of 40 ml medium. Half the medium was changed every 2 days and replaced with fresh medium with care taken to avoid removing cells.

After 7 days culture the cells were collected by centrifugation and then used in experiments. BMDM were maintained in RPMI plus 10% FCS and Pen/Strep for experiments.

The Murine fibrosarcoma cells, L929, spontaneously secrete into culture supernatant colony stimulation factor (M-CSF or CSF-1). The fibroblasts are grown in T162

flasks in 25 ml of DMEM medium. The supernatant is harvested 3 days after they reach confluency; filtered, aliquoted and stored at -80°C until the day it is added as a supplement to the growth medium. (The cells are split 1:30 and replated for future supernatant collection).

2.7 DNA extraction

For quantitative PCR cells of interest were cultured as described above. DNA was extracted using Direct PCR lysis reagent (Cell) (Viagen Biotech Inc, Los Angeles, California, USA). Briefly cells were lysed by the addition of lysis reagent containing proteinase K (Sigma). They were then incubated for 5 hours at 55^{o}C. 1µl of lysate was then used per PCR reaction as per manufacturer's recommendation.

2.8 Quantitative Polymerase Chain Reaction

In order to determine the deletion efficiency in alveolar macrophages of the cre recombinase in the Hif mice the presence of the Hif-1α gene in genomic DNA was compared between cre negative and cre positive animals. (Cramer et al 2003). In this assay the level of Hif-1α gene is compare to that of a reference gene (Haem oxygenase-1) which should have the same levels in both cre positive and negative animals.

This assay was performed with the help of Dr Matthew Sharp BRR Little France. HO-1 was chosen as the reference gene as this is the in-house positive control gene that the BRR facility uses to check all its PCR runs.

The PCR reaction was run on an ABPI7000 sequence detection system under standard realtime PCR conditions.

The reaction was performed in a final volume of 25µl

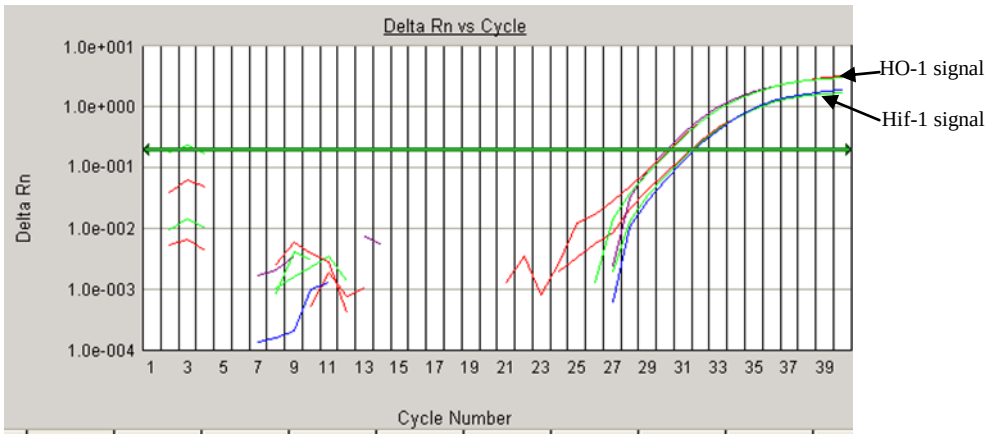
The reaction mixture comprise of

Abegene Absolute QPCR Rox mix (x2)	12.5µl
Primer/Probe Mix (x25)	1µl
Nuclease free water	10.5µl
DNA	1µ

The Hif-1 primers were designed using the Roche Universal Probe assay design centre (www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Probe 60 from the Universal probe set was used.

The HO-1 and Hif-1 assay were run in triplicate in separate wells. The CT for Hif-1 and HO-1 were determined and the fold difference in gene DNA was calculated using the Comparative CT method (ΔΔCT). Representative results for a wild type and knock out animal are shown in Figure 2.2.

a. Wild type Alveolar Macrophages



b. Hif-1α knockout macrophages

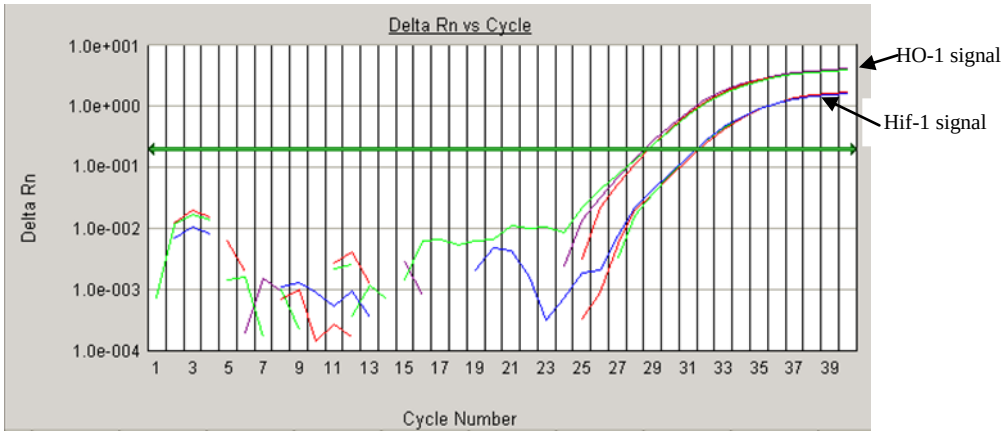


Figure 2-2 Representative PCR results from wild type (graph a) and hif-1 knock out alveolar macrophages. Each graph shows triplicate results for HO-1 (used as reference gene, left hand triplicate) and Hif-1 (gene of interest, right hand triplicate). It can be seen that there is an increase in the cycle number in the knock out animal before hif-1 product is detectable.

2.9 Genomic PCR

To confirm the presence of cre and the floxed alleles in our mice genomic PCR was performed. This was done on ear clip samples obtained by the BRR staff. DNA was extracted by the BRR facility staff using DirectPCR lysis reagent (Mouse ear) (Viagen).

For genomic PCR primers were designed by Professor R Johnson's team and they kindly supplied the protocols for the assays

Hif-1 PCR

Forward primer: TGC AAG TTG AAT AAC CGG AAA

Reverse primer: CTA GAG CCT GTT TTG CAC GTT C

The reaction mix used was as follows:

DNA	1µl
X10 buffer	5µl
MgCl2 (25mM)	2µl
dNTPs (10mM)	1µl
Forward (100pM)	1µl
Reverse (100pM)	1µl
Taq polymerase	0.5µl
Nuclease free H20	38.5µl
Total volume	50µl

The reaction conditions were

1minute 45seconds	94^{o}C
25 secs	94^{o}C
45 secs	57 ^{o}C repeat 36 times
60 secs	72 ^{o}C

Samples were run on a 2.5% agarose gel and visualised on the Versadoc imaging system.

Primers were synthesised by Eurofins MWG Raynes Park London. Taq DNA polymerase, deoxynucleosides (dNTPs) and nuclease free water were obtained from Promega (Promega Corp, Madison, WI, USA).

2.10 Statistical Analysis

Cell culture results were analysed by repeated measures ANOVA with Bonferroni's Multiple Comparison Test. The data were confirmed to be normally distributed using the D'Agostino & Pearson omnibus normality test.

Animal data sets were analysed with the Mann-Whitney test as normality could not be proven. All statistical tests were performed with GraphPad Prism v5.01 (GraphPad Prism Software Inc, La Jolla, California, USA). A p value <0.05 was taken as statistically significant.

For in vitro experiments cells were cultured in triplicate and the average was used as an individual result.

Chapter 3

Development of a Model of Acute Lung Injury

3.1 Introduction.

Major trauma is a common cause of Acute Lung Injury (ALI) recognised clinically as the Acute Respiratory Distress Syndrome (ARDS). At the time of injury trauma patients suffer a number of simultaneous insults which may lead to the development of ALI. These include hypovolaemic shock as a result of massive haemorrhage; tissue hypoxia secondary to impaired ventilation and reduced mixed venous PaO2 resulting in reduced arterial PaO2; long bone fractures and soft tissue injury; aspiration of oropharyngeal and gastric contents and visceral injury with release of potentially injurious contents (e.g. faecal matter). Furthermore if the patient survives, more insults including fluid resuscitation, artificial ventilation and surgery may precipitate ALI.

In order to study the role of these multiple factors it is necessary to first look at each in isolation in order to allow the researcher to gain a clear understanding of the pathogenesis of ALI. This led to the study a number of injurious insults which may lead to ALI in mice.

In general terms, models used in this field can be divided into 2 categories. They can either result in lung injury that mirrors that seen in humans, with histological evidence of alveolar neutrophilic infiltration and oedema and widespread epithelial and endothelial damage. Alternatively, models can involve the use of a stimulus that is thought to be involved in the generation of ALI and the early cell signalling effects can be studied. In these experiments there is often minimal evidence of lung injury,

either because the stimulus is of a subclinical severity or the animals are sacrificed at very early time points before injury is apparent. In this work the aim was to develop a model which clinically and microscopically results in a pattern of injury similar to the human disease as this confirms the relevance of the insult and potentially allows direct extrapolation of results to human studies.

Therefore, the aim of this piece of work was to develop a model of ALI which provides a suitable tool with which to study the role of myeloid cell Hypoxia-inducible factor 1 in the development of ALI.

In order to be worthy of further study the following conditions had to be fulfilled:

- the model must result in a disease process similar to that seen in patients
- the initiating insult must be clinically plausible
- the model must be reproducible by other groups

3.2 Fixed Volume Haemorrhagic Shock Model

Haemorrhagic shock (HS) has been reported to cause ALI in animal models in mice, rats and other species[157-159]. There are two possible approaches to the development of an HS model in animals. Either, the animal can have a variable volume of blood removed sufficient to reduce arterial blood pressure to a predetermined level for a fixed period of time (fixed pressure model) or a predetermined volume of blood is removed over a fixed period of time (fixed volume model) and in this case blood pressure is a dependent variable.

There are several advantages to the second model. Firstly, it more closely resembles the clinical situation[160] where a patient loses a proportion of their circulating blood volume but both the body's homeostatic mechanisms and subsequent medical efforts are directed at restoring blood pressure to near normal as quickly as possible. In addition it does not require more invasive monitoring of the animal which results in tissue trauma from the need to cannulate several major arteries.

3.2.1 Method

Male FVB/NJ mice were anaesthetised with 5% Isoflurane in oxygen before being restrained in a supine position on a heated mat, anaesthesia was then maintained with 1% Isoflurane in oxygen. A 29 gauge heparinised needle connected to a syringe was inserted into the heart by a sub-xiphisternal approach with the tip of the needle

directed towards the animal's left scapula. Gentle aspiration was maintained on the syringe until blood was aspirated. A predetermined volume of blood was then removed and the animal was allowed to recover in its cage. Recovery occurred within 5 minutes of the end of exposure to anaesthesia. Sham animals were anaesthetised and a cardiac puncture was performed but no blood was removed.

3.2.2 Results

In line with previous investigators the effect of removing 30% of calculated circulating blood volume (0.025ml/g body weight) was studied. A total of 27 animals underwent the 30% shock protocol. There was 1 death before the specified timepoint (3.7% mortality) which occurred a few minutes after the procedure without the animal recovering consciousness. Post-mortem examination revealed the cause of death to be massive haemothorax.

In line with Abraham's group[157] animals were allowed to recover for 1 hour after HS before sacrifice and lungs were removed for histological analysis. In none of the animals was there evidence of ALI when assessed by a Veterinary pathologist (Dr David Brownstein).

Abraham's group [157;161] has also reported an increase in lung myeloperoxidase (a measure of neutrophil content) and lung wet/dry weight ratio (a measure of pulmonary water content) in mice following HS. Consequently the effect of 30% HS on this two markers was studied at 4 hours, a later timepoint which has been reported as the peak injury by a group from the same laboratory. The combined results of two independent experiments are shown in Figures 3-1 and 3-2. At this 4 hour timepoint

there was no evidence of neutrophil accumulation or increased lung water content in the HS group. This was confirmed by histology from a further animal.

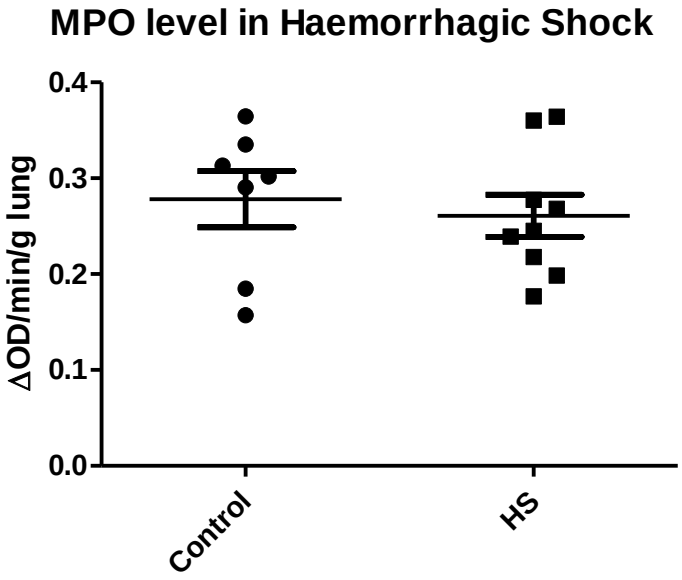


Figure 3-1 Effect of Haemorrhagic Shock (HS) on Lung Myeloperoxidase activity at 4 hours. Error bars represent Standard Error. p=0.54

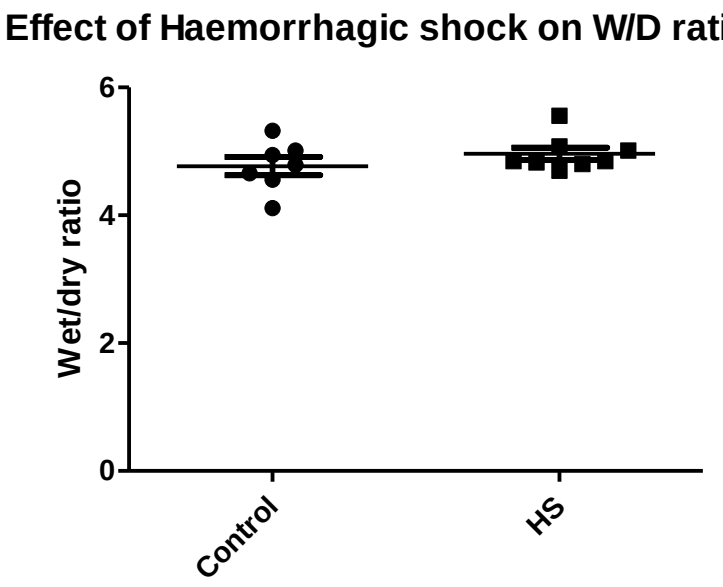


Figure 3-2 Effect of Haemorrhagic shock on lung wet/dry weight at 4 hours. Error bars represent Standard Error.p=0.29.

To determine if there was any evidence of tissue injury at a later timepoint or if a more severe insult could induce injury two further studies were undertaken. First after 30%HS mice were allowed to survive for 8,12,24,48 and 72 hours (n=1 at each timepoint) and the lungs removed for histological analysis. Again there was no quantifiable evidence of pulmonary injury at any timepoint.

Finally a group of animals underwent 40%HS, a more severe insult. This insult was associated with a 33% (3 of 9 animals) instant mortality (2 animals dying as a result of massive haemothorax and 1 undetermined cause). Animals were sacrificed at 1, 4 and 8 hours and 3 and 5 days. At no timepoint was there any histological evidence of pulmonary injury.

Therefore fixed volume survivable haemorrhagic shock at 30% or 40% of circulating blood volume did not lead to evidence of Acute Lung Injury over a 5 day recovery period.

3.2.3 Discussion

The reason for the differences between the observed data and Abraham's group [157;161-164] is unclear. The mortality quoted in their papers for their 30% HS protocol is 10-12%, significantly higher than the 3% seen in this study, suggesting that their insult is somehow more severe than that used here. Furthermore the exact nature of the pulmonary injury seen by Abraham's group is debatable. They show an increase in total lung MPO a measure of PMN number in lung tissue but this does not describe the cells' position. ARDS is characterised by a neutrophil rich alveolitis which Abraham's data do not support, their histology shows PMNs mainly in the pulmonary vasculature and some in the alveolar interstitium.

The observed difference may also relate to the animal facility used. These experiments were conducted in the University of Edinburgh's new animal facilities at Little France which have a significantly lower infectious agent load than the older facilities used and have resulted in the need to redesign other inflammatory rodent models (Dr P. Fitch personal communication) suggesting that the microbiological environment of the animals could have an effect on their inflammatory response. For example, the release of LPS from ischaemic bowel after the insult may play a role in the generation of ALI although this mechanism has been questioned[165] in a model of HS which failed to show any change in plasma endotoxin levels after HS. Alternatively, the differences may reflect physiological differences between the strains of mice used [166]. Abraham's group use BALB/c or C57 Black6 mice while this study used the FVB/NJ strain. This strain was chosen because of the potential for collaboration with other groups in the Centre for Inflammation Research which use

the FVB/NJ strain in other models of organ injury and is recognised to respond differently to other strains when exposed to other insults.

Other groups[167] have shown similar results to the observed data when studying models of haemorrhagic shock. They have found that HS “primes” the animal to a subsequent second insult such as systemic or local sepsis resulting in exaggerated pulmonary damage but that on its own it does not lead to evidence of pulmonary dysfunction either by histological assessment or by measuring pulmonary MPO levels or lung water content. It was felt that studying ALI initiated by a single insult was most likely to generate data that allowed dissection of the pathways involved. Therefore, this study did not further investigate the effect of a second insult on the development of ALI.

3.3 Hypoxia as a potential trigger for ALI

Lung hypoxia can be seen in critically ill patients. Trauma patients hypoventilate as a consequence of direct thoracic trauma or an altered level of consciousness following cerebral injury. Other groups of patients develop regional atelectasis with subsequent hypoxia as a result of pain, impaired cough or direct compression of lung parenchyma (e.g. pleural effusion) [168].

Hypoxia is a central component in the development of High Altitude Pulmonary Oedema [60-62] usually seen in mountaineers at heights over 3000m above sea level. These patients develop pulmonary oedema in the presence of normal left ventricular function, similar to the situation in ARDS.

Acute hypoxia has been reported to alter release of mediators such as cytokines, neuropeptides and surfactant components by pulmonary inflammatory and parenchymal cells which may result in lung injury.

As part of the study of the multiple hits that trauma patients are exposed to I therefore studied the response of mice to a brief period of severe hypoxia relevant to the clinical situation seen in trauma patients, where, following the insult during the period of pre-hospital care and before definitive airway management, usually in the Emergency Department in the UK, severe alveolar hypoxia can occur.

3.3.1 Method

FVB mice were exposed to hypoxia in a custom built Perspex chamber (Figure 3-3; Outer box 41x31x27cm; Inner box 31x21x20cm)) designed in accordance with Home Office requirements. An hypoxic gas mixture (94% Nitrogen, 6% oxygen; Cryoservice UK) was flushed through the chamber. Chamber oxygen levels were recorded using a paramagnetic oxygen analyser (Datex, UK), carbon dioxide was scavenged using soda lime and airflow was maintained by an electric fan. The chamber was filled with standard bedding and food and water were freely available.

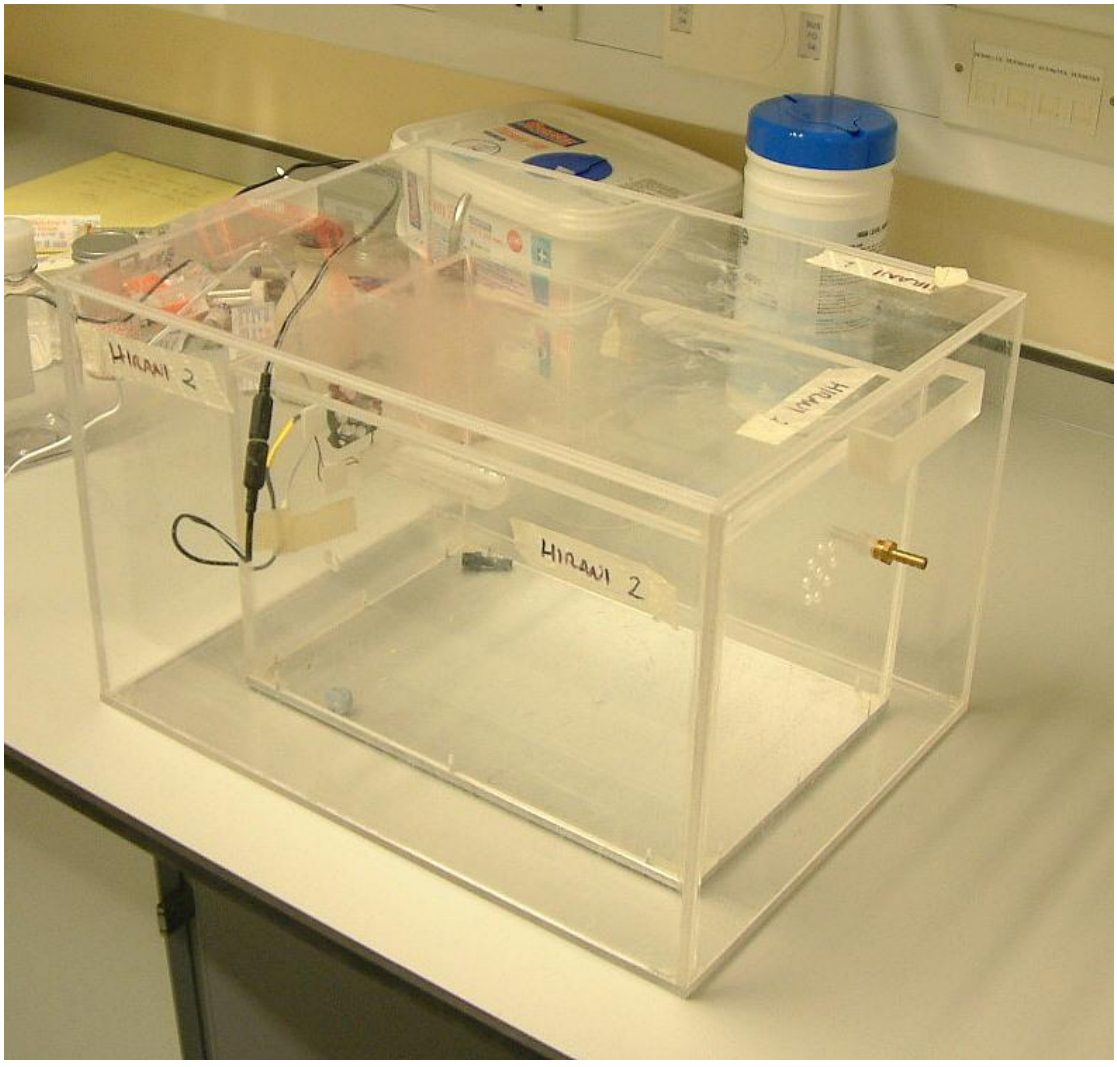


Figure 3-3 Chamber used for Hypoxic exposure

Using an initial flow rate of 15 litres/minute to purge the chamber an atmosphere of 8% oxygen could be rapidly reached and then maintained with a flow rate of 2l/min for the remainder of the experiment (figure 3-4)

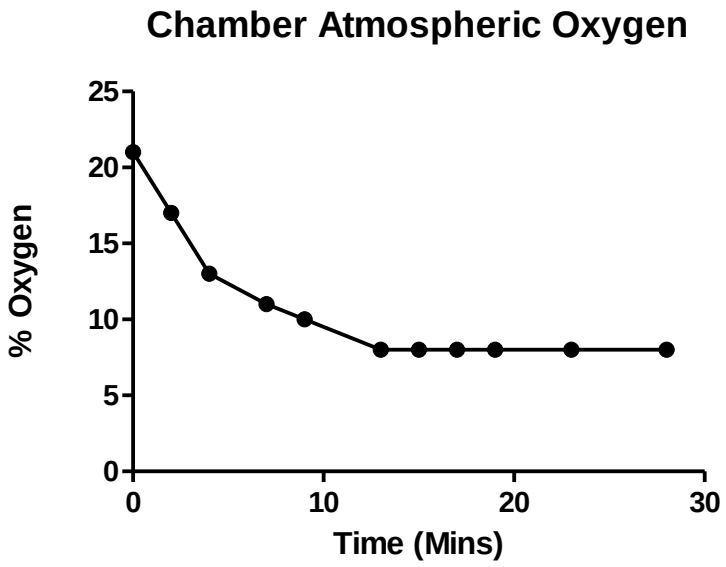


Figure 3-4 Representative recording of atmospheric oxygen from custom built chamber after flushing with hypoxic gas mixture.

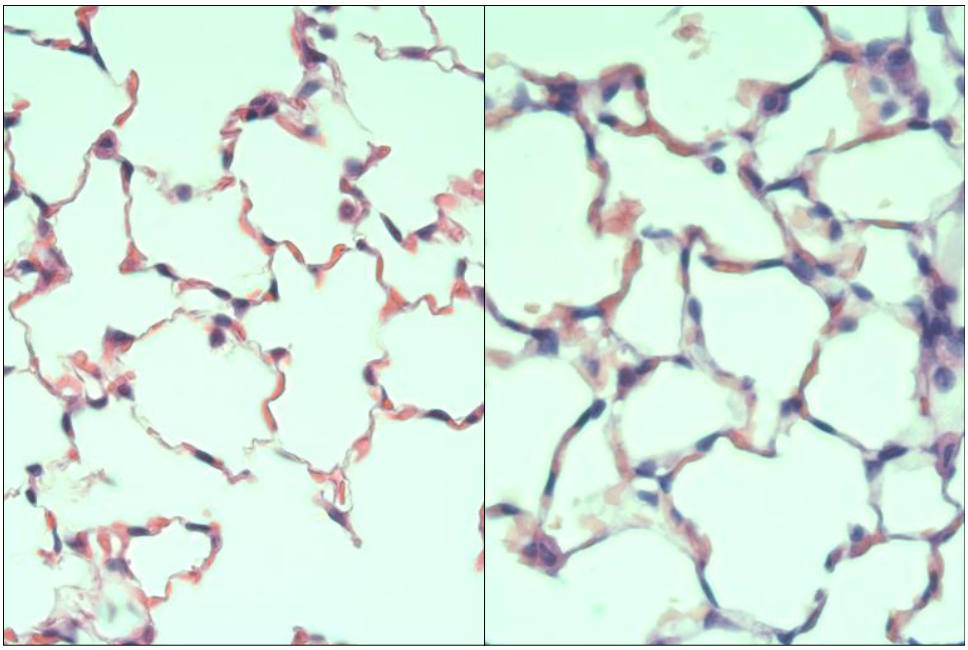
3.3.2 Results

Animals were exposed to acute normobaric hypoxia for 4 hours. With this they showed increased respiratory rates (range 130-200 breaths/min), piloerection, mild hunching and reduced activity. They continued to eat and drink. Mortality was 6% (n=17). Overall the severity of this procedure was moderate/severe as defined by the

Home Office. Therefore if further insults were to be added (e.g. haemorrhage) a higher atmospheric oxygen level would have to be employed.

Acute severe hypoxia has been reported to result in pulmonary neutrophil recruitment in some but not all studies. To assess neutrophil recruitment to the pulmonary compartment in this model pulmonary MPO was measured in some animals. 4 hours of acute hypoxia did not change the pulmonary MPO level compared to control animals (control 0.1043 ± 0.02396 N=4 v hypoxic 0.1301 ± 0.03392 N=4 p=0.55 graph).

Similar levels of acute hypoxia have been reported to cause pulmonary oedema in animal models when assessed by varying techniques. I used two measures to study the development of pulmonary oedema in this model. First lung wet/dry (W/D) weight ratio was used as a measure of total pulmonary water content which increases in pulmonary oedema. The W/D weight ratio was not different between control and hypoxic animals (control 4.790 ± 0.2035 N=7 v hypoxic 4.935 ± 0.2664 N=7 p=0.67 graph). In a separate group of animals vascular permeability was measured by the technique of Evans blue (EB) dye extravasation. Hypoxic animals showed a significant increase in EB extravasation over the 4 hour period (control E620 corr 0.8033 ± 0.1105 N=3 v hypoxic 1.557 ± 0.1299 N=3 p= 0.01). The increase in vascular permeability but lack of frank pulmonary oedema was confirmed by histology (figure 3-5) which showed an increase in alveolar septal thickness but no alveolar oedema. Histology also showed thrombus formation in pulmonary veins (figure 3-6) which has previously been reported in a murine model of hypoxia[169].



normal

8%Oxygen
Alveolar septal oedema

Figure 3-5 Normal Lung from Control animal and Alveolar Septal Oedema visible from animal exposed to 8% Oxygen for 4 hours.

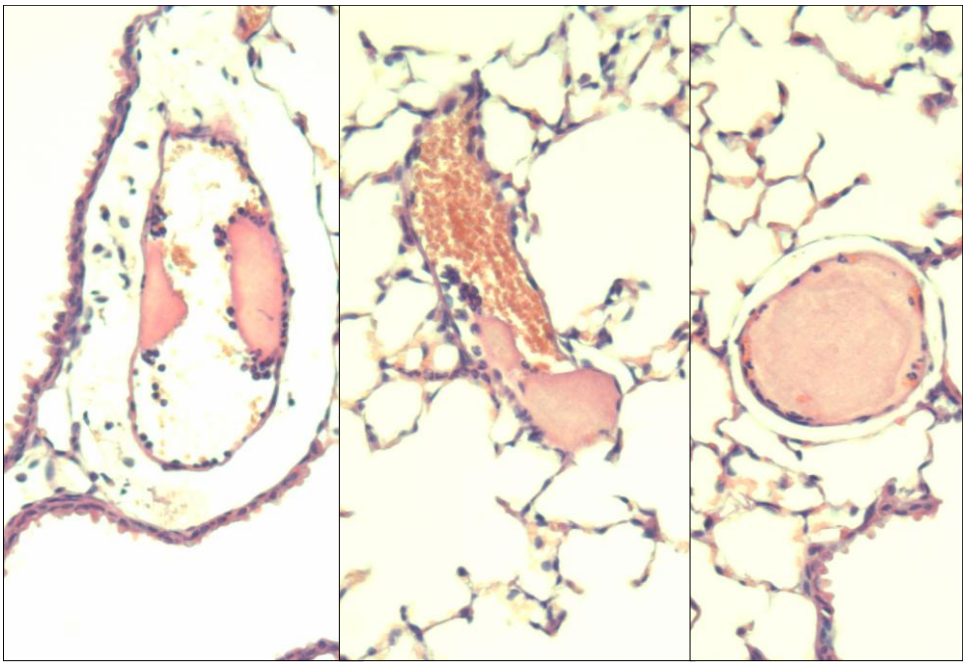


Figure 3-6 Microvascular Thrombi visible in pulmonary veins from animal exposed to 8% Oxygen.

3.3.3 Discussion

Alveolar hypoxia has been linked to the development of ALI. In my hands exposure to 8% oxygen for 4 hours leads to increased pulmonary vascular permeability in the absence of pulmonary neutrophilia. Others have reported a mild pulmonary inflammatory infiltrate in response to similar hypoxia in rats[65] although this finding has not been reproduced [170;171]. An increase in pulmonary vascular permeability has been a more consistent finding [65;172] while the development of pulmonary oedema has been reported by some [172;173] but not all authors [174;175]. Some of these differences may be species and strain related [154;176;177]. We chose a strain of mouse which most closely mimics the human cardiovascular response to hypoxia with a minimal hypotension response compared to other inbred mouse strains [154].

The mechanism of increased vascular leak is unclear. The increase may be due to increased endothelial permeability, increased capillary pressure or both. Acute hypoxia causes pulmonary arterial vasoconstriction which increases capillary pressure. However other substances such as an increase in the neurotransmitter Substance P and its receptor NK-1 have been reported in acute hypoxia and can result in increased vascular permeability and vascular pressure [174]. Other putative mechanisms include an increase in adenosine generation by pulmonary cells leading to increased endothelial permeability by a cAMP dependent pathway [172;178] or the release of pro-inflammatory cytokines by resident alveolar macrophages in response to hypoxia[63]. A further possibility is the effect severe hypoxia may have on lung biomechanics. Minko et al [173] have shown that hypoxia results in a change in pulmonary lipid composition, the major component of surfactant, and have proposed a role for this in the generation of lung injury. An increase in respiratory rate is a

consistent finding in studies and the mechanical effects of this have not been explored. Studies of any alteration in barrier function are required to assess the effects on the lung of operating at an increased frequency but smaller tidal volume, a situation seen in critically ill patients which may be a further “hit” to these patients’ lungs.

3.4 Systemic Endotoxin as a trigger for ALI

ALI is a frequent complication of severe sepsis and has a high mortality associated with its development. Sepsis can develop in critically ill patients either as their initial insult (e.g. meningococcal sepsis) or as a second insult such as peritoneal infection following abdominal trauma or bacteraemia associated with medical interventions including venous access devices. One of the proposed key triggers in the pathogenesis of sepsis is endotoxin, (Lipopolysaccharide [LPS]), a component of the cell wall of Gram negative organisms. With the absence of key features of ALI in the previous models we aimed to determine whether systemic administration of endotoxin would provide a useful model of ALI in our hands.

3.4.1 Method

Based on published data [179-181] a dose of 15mg/kg of *Escherichia coli* LPS serotype B127:08 (1mg/ml dissolved in PBS) was given by intraperitoneal (i.p.) injection to conscious mice in a final volume of 500µl. Doses of 10-25mg/kg have been shown to induce pulmonary neutrophil infiltration [182] in this strain. Similar doses have been shown to induce neutrophil infiltration of other organs [152]; stimulate the release potentially important cytokines [146] and induce metabolic changes [150] characteristic of sepsis with no observed mortality. Animals were then place back in their cage and allowed free access to food and water before sacrifice. Control animals received an injection of 500µl PBS i.p.

3.4.2 Results

Animals given i.p. LPS exhibited obvious features of systemic upset. Within 1 hour they displayed piloerection; a hunched posture; narrowed eyes and reduced spontaneous movement and self-care. These features persisted to the end of the experiment.

LPS administration resulted in a significant increase in pulmonary MPO levels compared to control animals (LPS group 6.008 ± 0.2952 N=4 v control 3.271 ± 0.3464 N=5; p=0.0007).

There was no difference in pulmonary wet/dry weight between groups at this timepoint (control 4.789 ± 0.07491 N=5 v LPS 4.929 ± 0.08286 N=4 p=0.25). Histology of sections showed neutrophil congestion and margination in alveolar capillaries and thickening of alveolar walls but there was no evidence of alveolar space exudate or cell infiltration (figure3-7).

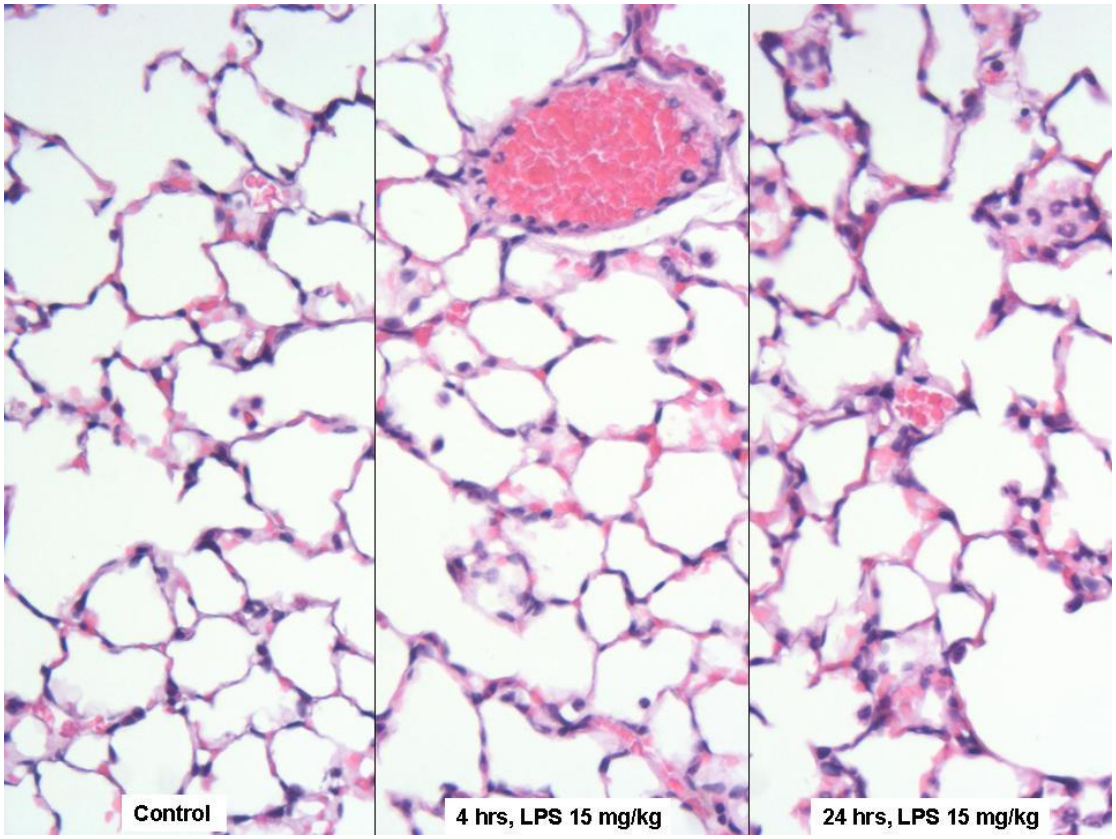


Figure 3-7 Histological Sections from Animals given intraperitoneal LPS and sacrificed at the indicated times.

3.4.3 Discussion

This experiment confirmed that systemic administration of LPS results in pulmonary neutrophil sequestration in the vasculature but at the timepoint studied showed no evidence of pulmonary oedema, alveolar haemorrhage or airspace inflammatory cell infiltrate important features of ALI were seen. Others have shown pulmonary oedema at this timepoint [157;181] accompanying an increase in pulmonary MPO content at similar and lower doses of LPS. However some investigators have failed to show pulmonary airspace neutrophilia over periods of up to 4 days [183;184] following systemic LPS. Interestingly Steinmuller et al [184] showed a large, persistent increase in bronchoalveolar lavage MIP-2 levels despite a lack of neutrophil alveolitis suggesting that even though systemic LPS can lead to high local levels of neutrophil chemoattractants the injury generated may not be enough to stimulate neutrophil migration into the airspace.

The animals given this dose of LPS exhibited marked illness behaviour and signs of distress. Given the nature of the observed pulmonary neutrophilia and the lack of consistent alveolitis found by other investigators it was felt that the burden placed on the animals during the experiments did not justify continuing to use this model and I moved to a model of pulmonary inflammation induced by instillation of LPS into the lung directly.

3.5 Pulmonary Instillation of LPS as a model of ALI

The direct exposure of rodent lung to injurious stimuli including LPS and IgG immune complexes has been used to model ALI [185;186]. Techniques to deposit the agent into the lung include inhalational exposure by nebulising the agent [187]; intranasal administration and subsequent inhalation [188]; aspiration of the agent from the oropharynx or direct tracheal instillation either following surgical tracheotomy [189] or intubation of the trachea through the vocal cords [190]. Although it may not reflect the entire complexity of ALI, instillation of LPS has been widely used as a model of ALI because of its clinical relevance with the frequently seen important role of Gram negative bacteria in initiating lung injury and the resultant injury exhibiting the key features of ARDS with a neutrophil rich alveolitis developing with damage of the alveolar-capillary barrier resulting in a protein rich alveolar exudate.

Two methods of depositing LPS into mouse lung were studied in this work.

3.5.1 Oropharyngeal aspiration model method

Aspiration of material from the oropharynx is used in the CIR as a method of inducing ALI. Animals are anaesthetised by exposure to 5% Isoflurane and then suspended by their incisors from a custom built stand (figure 3-5). The animals tongue is then grasped and pulled forward to try and prevent deposition of the agent in the oesophagus. A 50µl volume of solution in then deposited in the back of the oropharynx and as the animal breathes the solution is aspirated into the trachea and

deposited in the lungs. The animal is then allowed to recover. With this technique the period of anaesthesia is short, usually 2-3 minutes total.

3.5.2 Tracheal intubation method

The second technique used was based on a method of endotracheal intubation of rodents [191]. The mouse was anaesthetised with an i.p. injection of Avertin. It was then suspended by its top incisors on the same support stand and the trachea was trans-illuminated by a cold light source (figure 3-6). The mouth was held open by a weight suspended from the lower incisors and the tongue was pulled forward. A sterile guide wire (0.8mm OD, Vygon UK) was then inserted into the trachea under direct visualisation. A 22G cannula was then passed over the wire and into the trachea. The guide wire was then removed and the animal allowed to breathe through the cannula. (Figures 3-7 to 3-9) Correct placement of the cannula could be confirmed by transient obstruction of the end of the cannula and the animals breathing pattern would be seen to change. 50µl of solution was then pipetted into the cannula and the mouse would inhale the solution. The cannula would be removed after complete inhalation of the solution (usually about 10 breaths) and the animal would be allowed to recover from anaesthesia. The period of anaesthesia here would usually be 30-45 minutes.

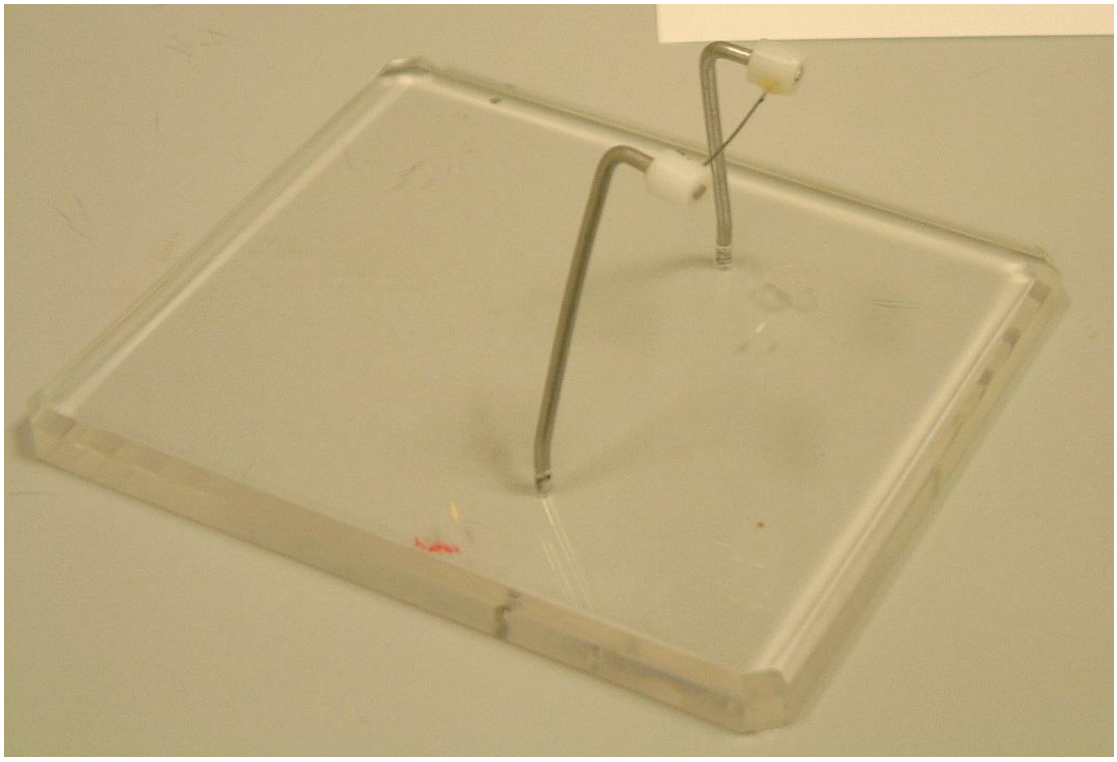


Figure 3-8 Support stand used for Pulmonary Instillations.

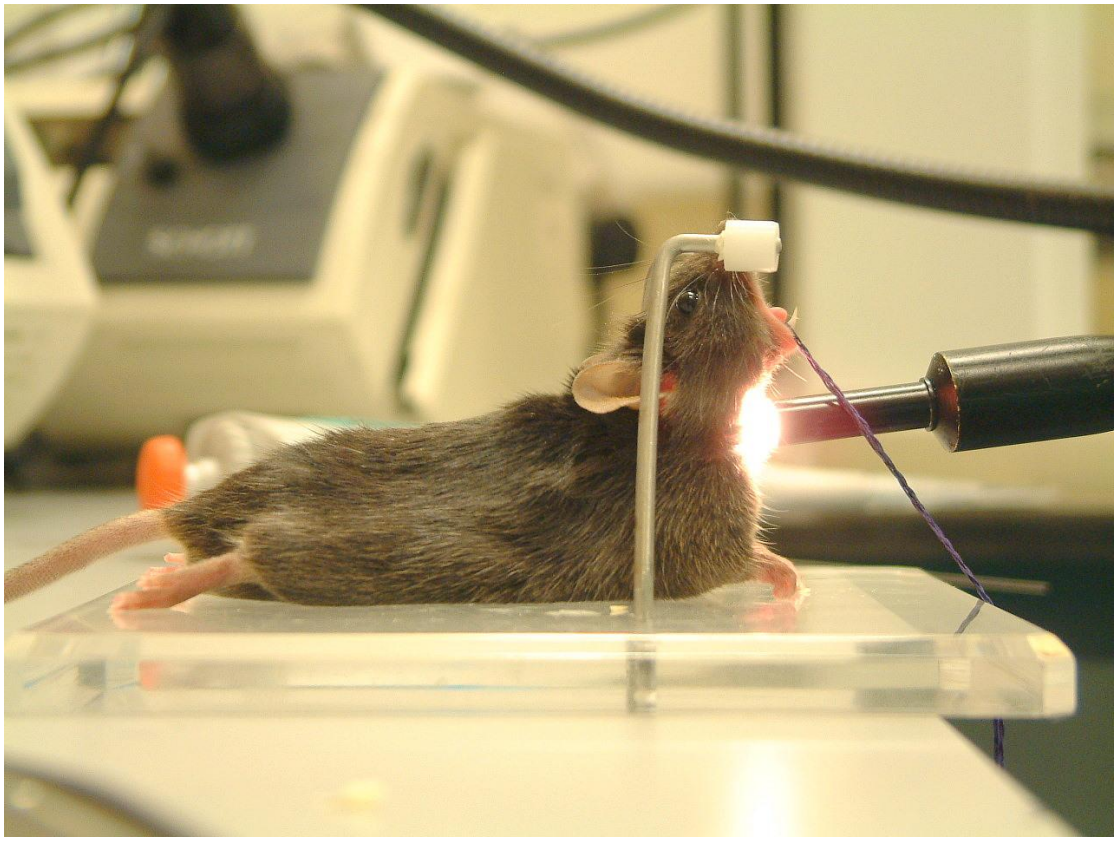


Figure 3-9 Mouse positioned to allow tracheal intubation under direct visualisation.

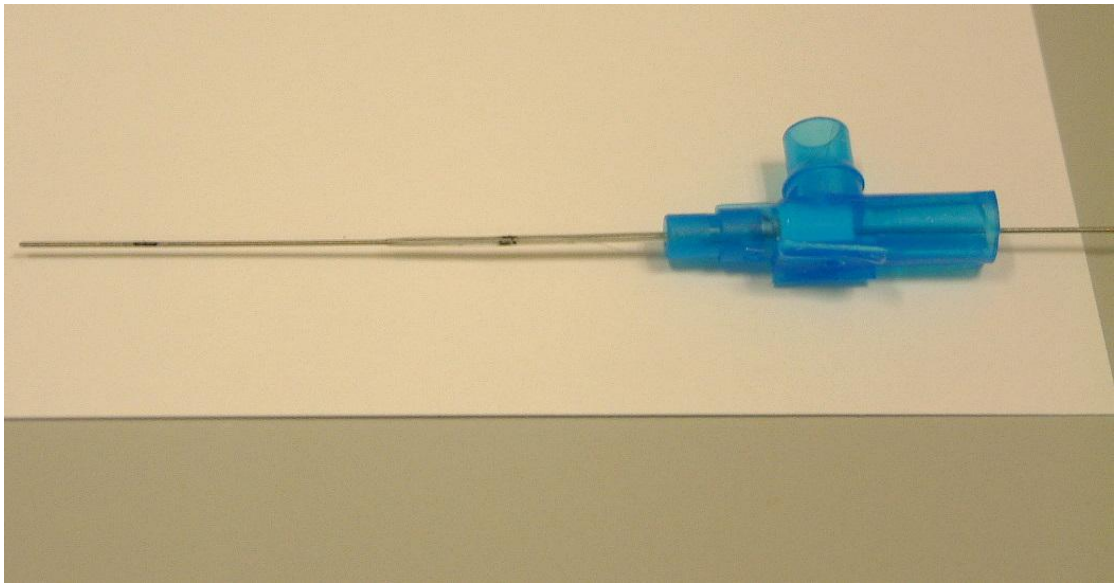


Figure 3-10 22G cannula loaded over guidewire.

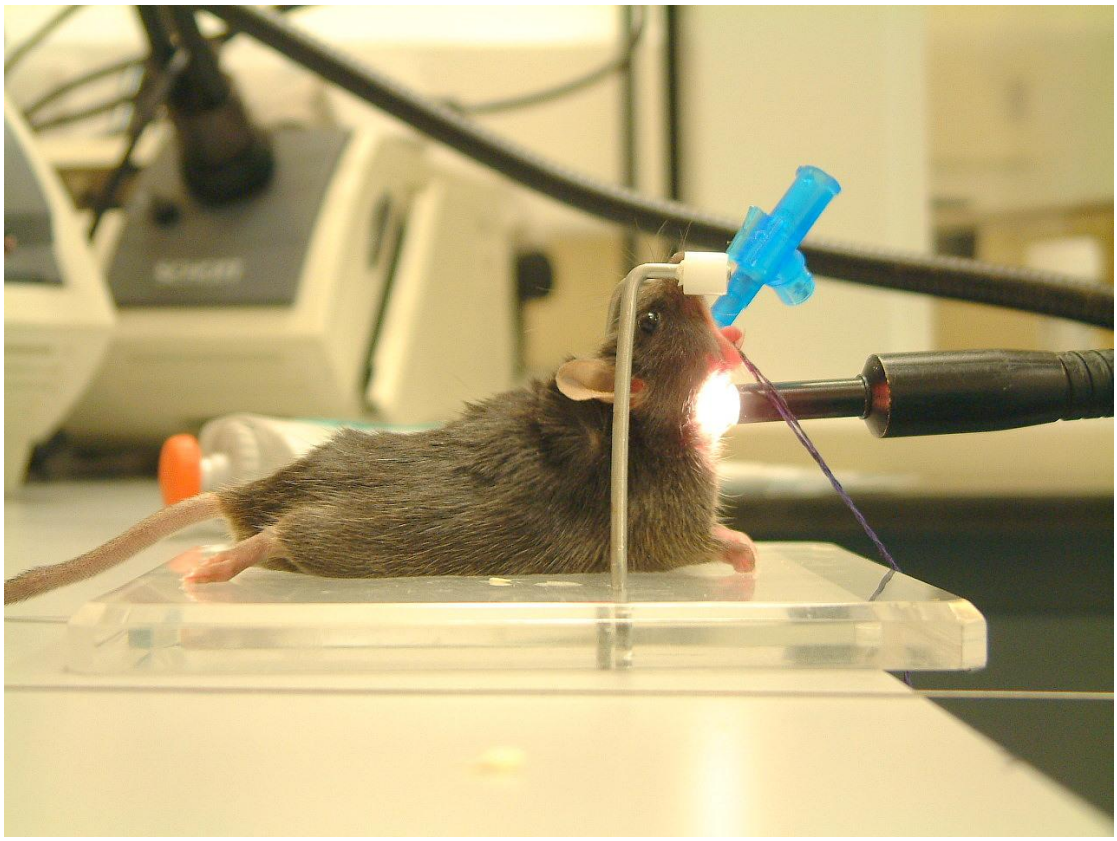


Figure 3-11 Mouse with trachea intubated by cannula.

3.5.3 Results of oropharyngeal aspiration and tracheal intubation methods

Initially the oropharyngeal technique was used. Instillation of 50μg of LPS in 50μl of saline resulted in an intense neutrophilic alveolitis at 6 hours. BAL total cell number increased from 218800 ± 45480 (N=4) in saline instilled animals to 516900 ± 61860 (N=4). This increase was due to an increase in alveolar neutrophil number (control 0 v LPS 438294±68448) and a decreases in alveolar macrophage number (saline 218800 ± 45480 v 74140 ± 9996) similar to previous authors findings (Asti et al 2000). Further as a measure of interstitial neutrophil accumulation lung homogenate MPO was measured after BAL and was also increased in LPS treated animals (saline 0.7430 ± 0.1024 v LPS 5.233 ± 0.3568 ΔOD/min/g lung $p< 0.0001$). There was no difference in BAL total protein at this time point (saline 294.1 ± 39.35 v LPS 324.1 ± 42.66 $p=0.6$).Thus direct injury of murine lung by instillation of LPS results in a neutrophilic alveolitis similar to ARDS. It also results in an increase in parenchymal neutrophils that are not accessible by BAL at this time point and likely represents cells trafficking from the vascular compartment to the alveolar space (Reutershan et al 2005)

However, when this experiment was repeated again a different pattern of injury was seen. On this occasion there was no difference in total cell number (saline 297800 ± 145000 N=4 v LPS 376000 ± 165100 N=3, $p=0.7$); macrophage number (saline 101900 ± 9475 N=4 v LPS 58340 ± 17290 N=3, $p=0.06$) or PMN number (saline 195600 ± 151000 N=4 v 401600 ± 238900 N=3, $p=0.5$). The lack of difference was due to an increase in PMN in the saline treated group as there was no difference between PMN number in LPS treated animals (expt 1 438300 ± 68450 N=4 v expt 2

401600 ± 238900 N=3, p=0.87). Study of cytospins of BALS from saline treated animals revealed the presence of a spectrum of both Gram positive and negative bacteria suggesting that the aspiration technique allows the carriage of oropharyngeal flora into the lung during the aspiration leading to an alveolitis in control animals.

Hence although this technique demonstrates that inhalation of LPS leads to ALI the magnitude of the effect could be hidden by the confounding effect of aspiration of the resident oropharyngeal flora, therefore a direct intratracheal instillation technique was developed.

Initial attempts at intratracheal instillation either using a blunted 25G needle attached to a syringe or a fine gel-loading pipette tip attached to a pipette were unsuccessful because of an inability to identify successful tracheal intubation and were abandoned.

The technique described above using a modified Seldinger technique which is used for many medical procedures proved easy to learn, reliable and avoided complications seen with other techniques for direct instillation such as mild alveolitis following surgical tracheotomy [192]. Preliminary studies using instillation of Evans blue dye as a marker of fluid deposition confirmed tracheal deposition and pulmonary spread of solution in animals occurring rapidly before sacrifice after 5 minutes (Figure 3-11). They also showed no deposition of any blue dye in the oropharynx therefore avoiding the possibility of instillation of resident bacteria into the lungs of animals.

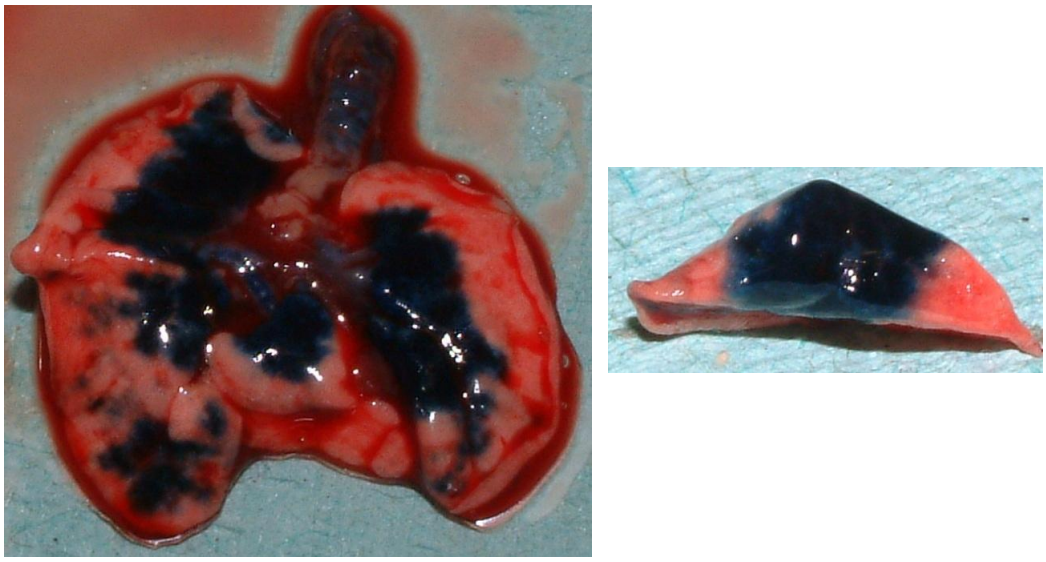


Figure 3-12 Lung Block and Section of Lung showing Deposition of Evan's blue dye throughout pulmonary parenchyma within 5 minutes of instillation.

This technique therefore became the standard method for instilling LPS into the lungs of animals for the remainder of the project. In a group of C57 Black 6 mice studied at 24 hours instillation of LPS resulted in an intense neutrophilic alveolitis (Total cell count Saline 196500 ± 9186 N=4 v LPS 1787000 ± 132400 N=5 $p< 0.0001$; PMN count Saline 922.8 ± 347.9 N=4 v LPS 1507000 ± 122900 N=5 $p< 0.0001$) in treated animals associated with a loss of alveolar capillary integrity reflected by an increase in BAL protein content (Saline 157.9 ± 6.227 N=4 v LPS 468.5 ± 76.92 N=5 $p=0.0093$). Histology confirmed the movement of neutrophils into the alveolar space. This was accompanied by the development of a protein rich eosinophilic exudate and in some alveoli the movement of erythrocytes into the alveolar space as a result of the disruption in the alveolar-capillary barrier integrity (Figure 3-13).

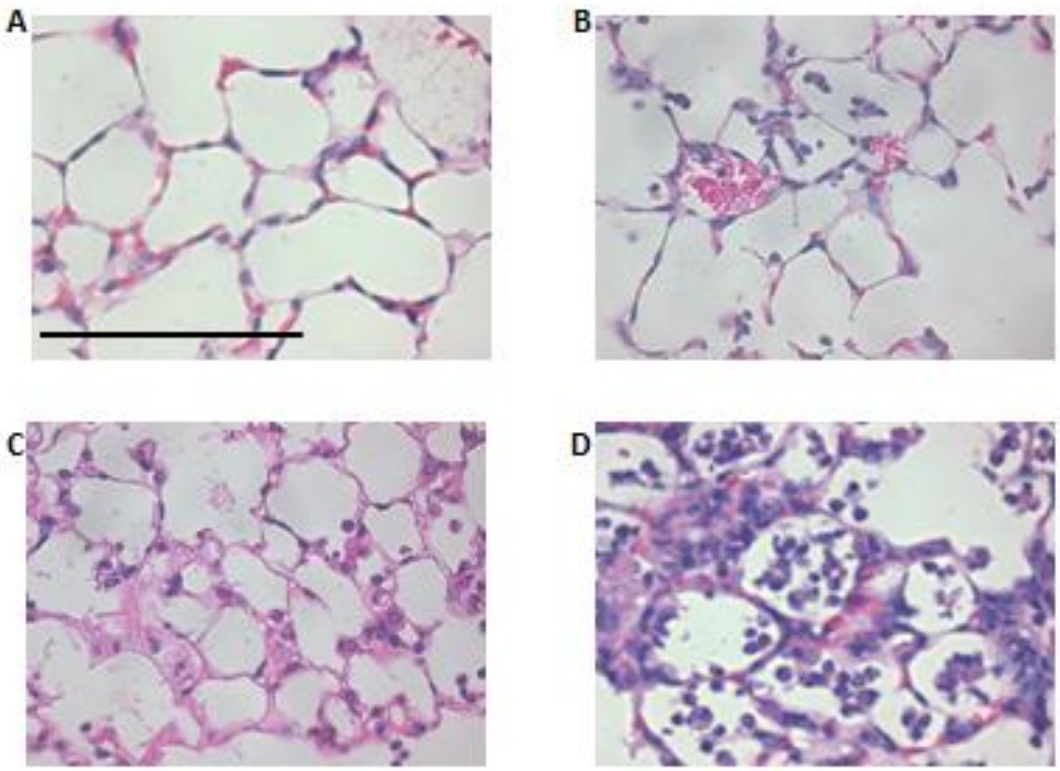


Figure 3-13 Intratracheal administration of LPS results in lung injury. Picture A Normal mouse lung. Pictures B 24 hours post-LPS instillation there is the development of neutrophilic alveolitis with free erythrocytes visible in one alveolus. Picture C Again at 24 hours post-instillation in some alveoli there is the accumulation of a proteinaceous exudate as a result of increase permeability of the alveolo-capillary barrier. Pictue D At 5 days post instillation the intense neutrophil infiltrate persists and there is thickeniong of the alveolar septae. x200 Graticule=100µm.

3.5.4 Discussion

This study has confirmed that direct instillation of LPS by two techniques results in a pattern of lung injury in mice that reflects the pathological findings of ARDS in man. Similar results have been shown by other investigators [187;192-194].

The advantage of the adopted tracheal intubation technique over the other techniques tried here and described in the literature include: absence of contamination of the instillate with oropharyngeal bacteria; minimal trauma compared to surgical tracheotomy; lack of any pulmonary barotrauma resulting from forced expulsion of liquid from the syringe or pipette as the animal inhales the liquid through the cannula and most importantly ease and reproducibility of the technique.

The dose of LPS routinely used in this study is 50μg in 50μl of Saline. This is at the higher end of the dose range used in the literature although higher doses have been reported [148]. Liaudet et al [193] used this dose and were able to show a reduction in injury with concomitant administration of inosine suggesting that the dose does not represent an overwhelming supramaximal stimulus. However, it does represent a severe injury leading to some of the changes seen in human samples from patients with ARDS.

It is appreciated that no single animal model fully represents the changes seen in humans however, the intratracheal endotoxin model does mimic a number of the changes seen: pulmonary neutrophil infiltration with movement of neutrophils from the intravascular space to the interstitium and then into the alveolus; accumulation of protein rich fluid in the alveolar space (although hyaline membranes were not seen in this piece of work); intra-alveolar haemorrhage and vascular congestion.

3.6 Conclusion

This chapter has been concerned with the development of an animal model of Acute Lung Injury. To be suitable the model needed to fulfil several criteria:

- the model must result in a disease process similar to that seen in patients
- the initiating insult must be clinically plausible
- the model must be reproducible by other groups

It was also preferable that the model relied on a single insult (e.g. haemorrhagic shock rather than haemorrhagic shock and resuscitation) so that the pathways resulting in organ injury can be more easily studied in future work.

The results of the work are summarised in table 3-1.

Table 3-1 Summary of Injury Pattern seen after differing Insults

	Injury pattern		
Model	Histological injury*	Alveolar neutrophil infiltration	Evidence of increased alveolar permeability
Haemorrhagic shock	No	No	No
Acute Hypoxia	Yes	No	Yes
Systemic LPS	Yes	No	No
Intratracheal LPS	Yes	Yes	Yes

Histological injury*- in this body of work this refers to a microscopic pattern of injury which is composed of some of the features seen in human ALI. As discussed in the individual model sections this includes the presence of alveolar neutrophils; interstitial neutrophil accumulation; alveolar septal oedema; microvascular thrombi, accumulation of alveolar proteinaceous fluid and intra-alveolar haemorrhage [195].

Using a number of models reported to generate ALI in the literature I found that direct tracheal instillation of LPS fulfilled the above criteria. It results in a neutrophilic alveolitis with an associated loss of alveolar-capillary barrier function. Aspiration of Gram negative bacteria or their cell wall components is a common insult in patients with ARDS and delivery of LPS to the alveolus has been reported by a number of groups in a range of species (including mouse and rat) to lead to ALI.

The lack of injury following some of the reported techniques and the possible reasons for this has already been discussed. Future work could study other mouse strains to see if they develop ALI in response to those insults in our laboratory. However, the lack of tissue injury seen by other authors (e.g. Ayala 2002 [167]) in similar models supports the observed data.

The hypoxic exposure model does result in the development of increased pulmonary vascular permeability and may in the future be useful in studying the response to severe hypoxia in settings such as adaptation to high altitude exposure but is not suitable for the current piece of work.

The usefulness of the model of LPS injury is increased by the ability to replicate it in vitro by exposing cultured cells to the same insult and then studying the response of individual cell types. Such work helps to identify the source of important mediators that may result in tissue injury and test possible therapeutic interventions.

Using both in vivo and in vitro techniques the remainder of this thesis will be concerned with the role of alveolar macrophage Hif-1 in the development of ALI in mice.

Chapter 4

Hif-1 mediation of the inflammatory response in alveolar macrophages

4.1 Introduction

The alveolar macrophage is a key cell in the detection of and response to pulmonary pathogens. It is located in a prime position to interact with pulmonary insults and functions as part of the lung innate immune response (phagocytosis; release of nitric oxide; secretion of cytokines) and also as part of the acquired response acting as an antigen presenting cell[196].

The importance of alveolar macrophages in the generation of ALI has been confirmed in a range of injury models by selective depletion of these cells which results in reduced lung injury[76;77;197;198].

The alveolar macrophage is exposed to alveolar oxygen levels (approximately 14kPa [199]) and therefore is unique among tissue macrophages in residing in a niche where oxygen is abundant, the intracellular pO_2 of the alveolar macrophage is however unknown. Furthermore, in ALI the macrophage may be exposed to a reduced pO_2 and this may modulate its responses.

Two key mediators in the development of ALI produced by alveolar macrophages are Nitric Oxide (NO) and Tumour Necrosis Factor-α (TNF). There have been studies on the effect of varying oxygen levels on the production of NO and TNF in a range of macrophage cell lines [200-202] and primary cells [82;83] but the concurrent production of both from a murine alveolar macrophage line (MH-S) exposed to severe hypoxia has not been studied. In addition, the effects of pharmacological manipulation of Hif-1, by a prolyl-hydroxylase inhibitor, on alveolar macrophage responses to injurious stimuli have not been reported. CXCL-12 was the first chemokine to be shown as being regulated by Hif-1α. It is chemotactic for a range of inflammatory

cells and has been shown to have a role in wound healing and renal ischaemic responses. It may also have a role in pulmonary inflammatory responses. The in vitro synthesis of CXCL-12 has not been described for the MH-S line.

The hypothesis being considered in this chapter was that “Hif-1 mediates the pro-inflammatory response to injury by alveolar macrophages”.

To test this hypothesis the aims of this chapter were to:

-study the effects of varied oxygen concentrations on NO and TNF production by a murine alveolar macrophage cell line

-study the effect of a prolyl-hydroxylase inhibitor, dimethyloxalylglycine (DMOG), on concurrent NO and TNF release

-describe the secretion of CXCL-12 by MH-S cells.

4.2 Results

4.2.1 Establishment of optimal cell density and dose of LPS.

In order to determine an appropriate cell density to be used an initial experiment was performed in a 24 well plate with cells used at 3 different densities. LPS was added at 1μg/ml to some wells and cells were cultured for 24 hours. The production of nitrite is shown in Figure 4-1.

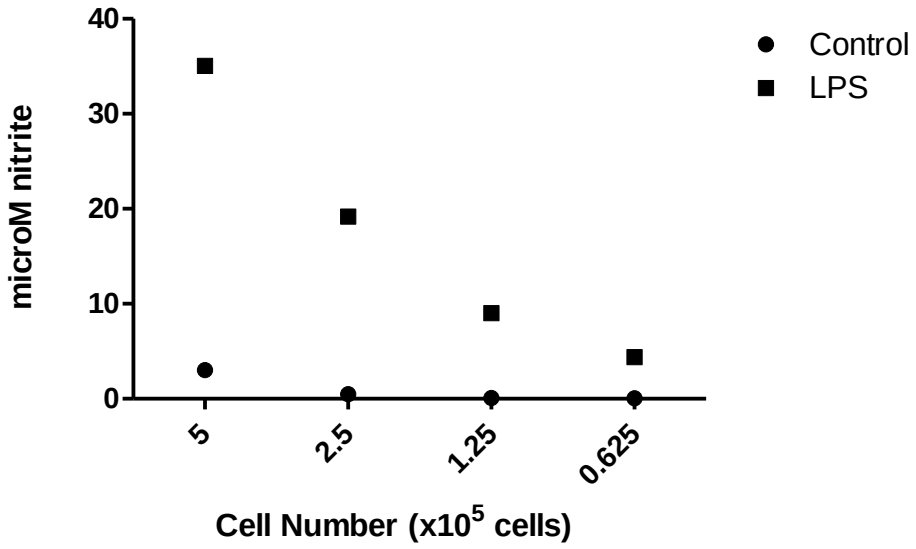


Figure 4-1 Nitrite levels in supernatants from cultured MH-S cells Results from triplicate wells. Mean result shown.

On the basis of this experiment it was decided that $2x10^5$cells per well in 1ml of medium was a suitable density. This balanced the need for a robustly detectable range of nitrite production with using an economical number of cells per well.

Subsequently an experiment was performed using the established cell density to determine a suitable dose of LPS to use. The result is shown in Figure 4-2.

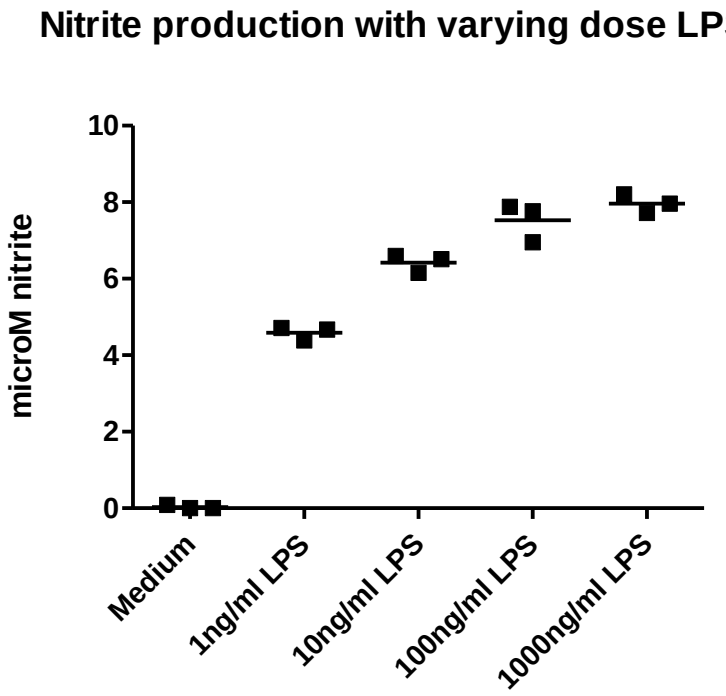


Figure 4-2 Nitrite generation by MH-S cells in response to varying doses of LPS. Results from triplicate wells plated at 200,000 cells per well. Triplicate results shown.

On the basis of this experiment and preliminary data which showed an augmented response with DMOG (not shown) a dose of 1μg/ml LPS was chosen as the standard

dose of LPS used. The production of nitrite and dose of LPS chosen corresponded well to other investigators results published in the literature[203].

Finally, to determine an optimal time for measuring nitrite production experiments were performed collecting supernatant at 1,2, 6, 24 and 48 hours after addition of LPS (Figure 4-3).

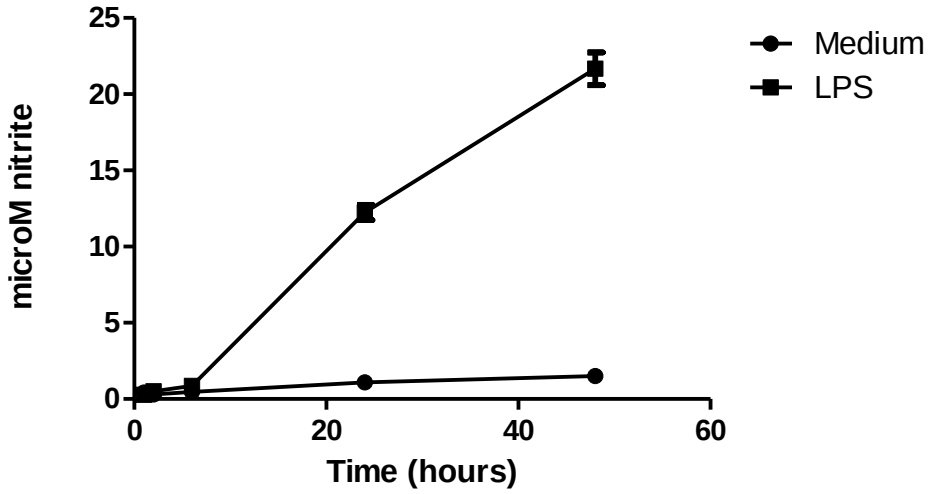


Figure 4-3 Timecourse for Nitrite generation by MH-S Cells. Error bars represent Standard Error. Results for triplicate wells shown.

It can be seen that nitrite levels first became easily detectable at 24 hours and continued to increase in wells given LPS for up to 48 hours. However, using LDH as a measure of cell death it was apparent that by 48 hours there were high levels of cell death in wells given LPS (Graph 3-4), approaching 50%. Therefore 24 hours was chosen for the remaining experiments.

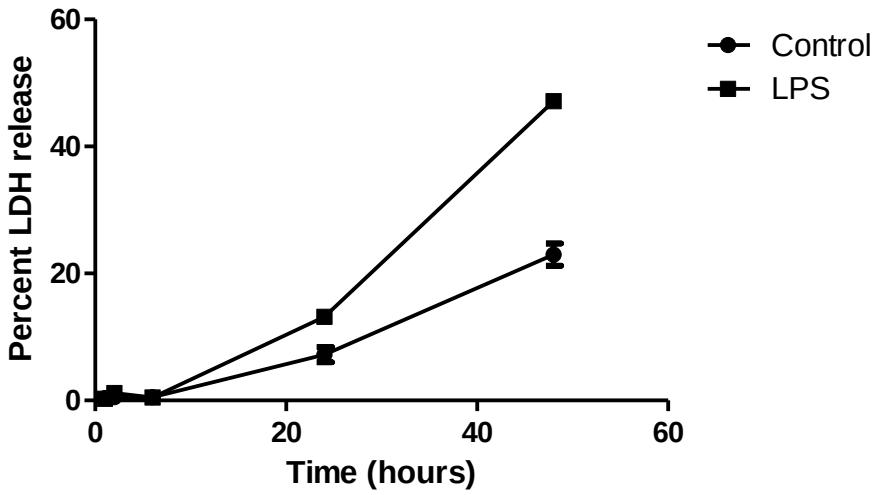


Figure 4-4 Cell death assessed by LDH release in response to LPS. Error bars represent Standard Error. Results for triplicate wells shown.

4.2.2 Effects of hypoxic culture on MH-S response to LPS

To test the effect of hypoxic exposure on the response of MH-S cells to LPS it was decided to expose the cells to an atmosphere of 0.5% oxygen (5%CO_2/94.5%N_2), a severe level of hypoxia which has been shown to induce Hif-1α protein stability in macrophages (Blouin et al [142] used 1%). In addition cells were also studied at 14% atmospheric oxygen, corresponding to alveolar levels, to compare the effect of this with traditional culture conditions at room air (21% oxygen) which can be viewed as hyperoxic.

The results of three independent experiments performed in triplicate are shown in Figure4-5.

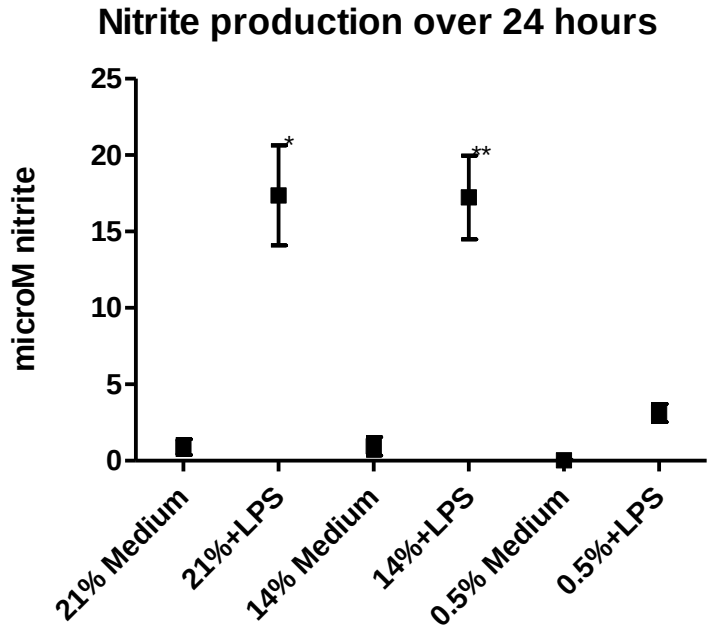


Figure 4-5 Generation of Nitrite in response to LPS in MH-S cells cultured in varying ambient oxygen levels. Error bars represent Standard Error. * p<0.001 compared to 21% oxygen. **p<0.001 compared to 14% oxygen. Result of 3 separate experiments with triplicate wells.

Minimal nitrite was produced by resting cells at all oxygen concentrations. As expected cells cultured in room air given LPS generated large amounts of nitrite over the 24 hour period. There was no difference in nitrite generation between cells given LPS cultured at 21% or 14% (17.37 v17.27 μM p=NS) so all future experiments were performed solely with control cells cultured at 21% oxygen. Cells cultured at 0.5% oxygen for 24 hours produced significantly less nitrite (3.126+/-0.6 μM v 17.4+/-3.7) compared to cells cultured at 21% with LPS.

TNF levels in supernatant were measured for cells cultured at 21% and 0.5% both in the presence and absence of LPS (Figure 4-6).

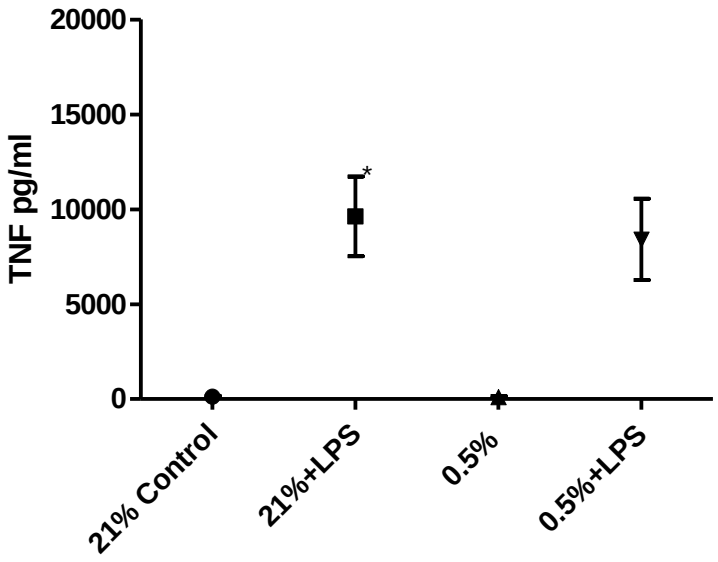


Figure 4-6 TNF release by MH-S cells in response to LPS in Normoxia or hypoxia (0.5% Oxygen). Error bars represent Standard Error. *p<0.05 compared to 21% oxygen. Result of 3 separate experiments with triplicate wells.

Again as expected resting cells produced minimal levels of TNF (21% 125+/-42pg/ml) and there was no effect with culture at 0.5%. Exposure to LPS resulted in the production of large amounts of TNF again similar to results obtained in a previous study [203]. There was no significant effect on TNF production in response to LPS when the cells were cultured at 0.5% oxygen (21%+LPS 9630 ± 2098 N=3 v 0.5%+LPS 8417 ± 2136 N=3).

4.2.3 Effect of hyperoxic culture on MH-S response to LPS

Patients with lung injury are treated with supplemental oxygen. It was therefore decided to study the effects of 24 hours of hyperoxic exposure on cells. 60% oxygen was chosen as an appropriate level of hyperoxia as this represents a clinically achievable level of oxygen in spontaneously ventilating patients.

As can be seen from Figure 4-7 hyperoxic culture had no significant effect on nitrite production either in cells at rest or exposed to LPS.

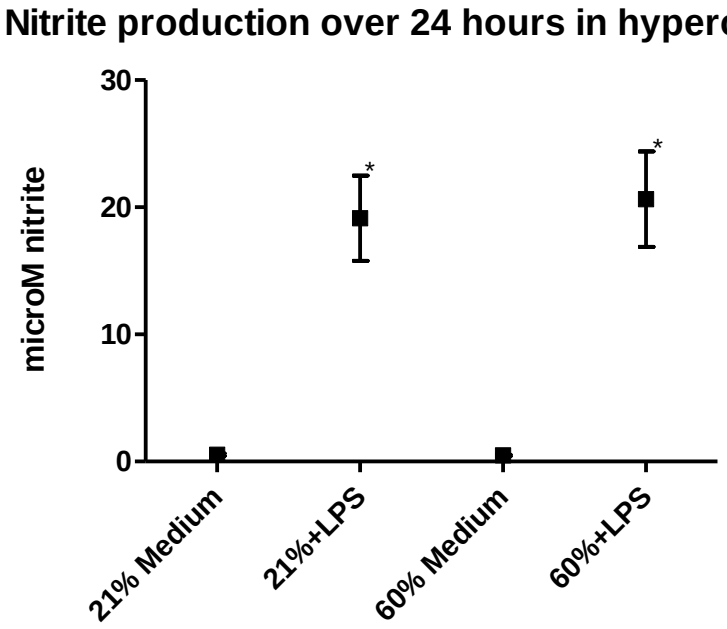


Figure 4-7 Nitrite release by MH-S cells in normoxia or hyperoxia (60% oxygen) in response to LPS. Error bars represent Standard Error. Result of 3 separate experiments with triplicate wells. *p<0.0001 compared to 21% medium.

There was similar levels of LDH release in all conditions (figure 4-8) suggesting that viability was not affected.

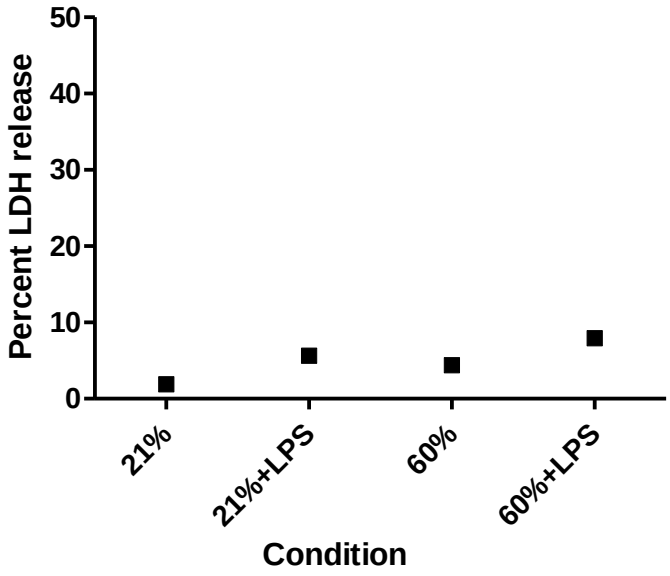


Figure 4-8 LDH release by MH-S cells in normoxia and hyperoxia (60% oxygen) in the presence or absence of LPS. Result of 3 separate experiments with triplicate wells. p>0.05 for all conditions.

The expression of TNF in this series of experiments was measured in 2 sets of the 3 experiments. There was no difference in TNF production between cells grown at 21% and 60% oxygen. (Graph 4-9)

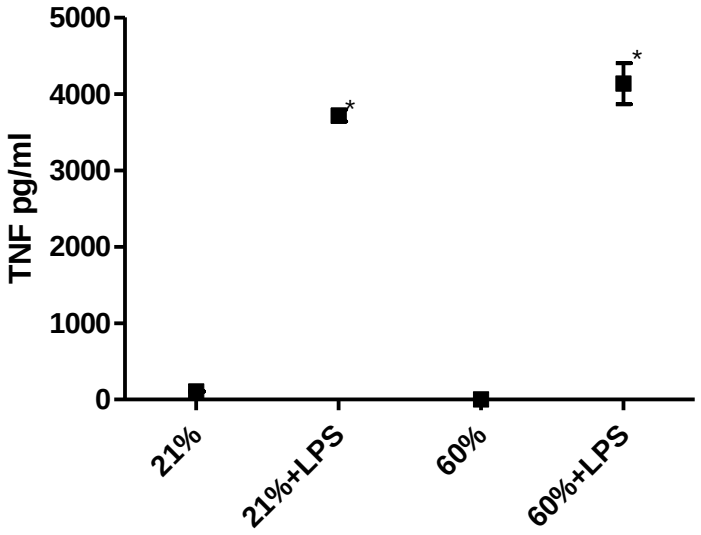


Figure 4-9 TNF release by MH-S cells in normoxia or hyperoxia (60%) in response to LPS. Error bars represent Standard Error. Result of 3 separate experiments with triplicate wells.* $p<0.0001$ compared to 21% oxygen.

4.2.4 Reoxygenation rapidly increases nitrite levels in hypoxic culture

Having shown that hypoxic culture resulted in a reduction in the production of nitrite following LPS exposure and that hyperoxic culture had no significant effect on nitrite production it was decided to study the effect of a period of hypoxia (18 hours) followed by a varied period of reoxygenation. Again 60% oxygen was chosen as the reoxygenation atmosphere as it was felt to represent the clinical situation of interest where a patient has been unwell for a period of time and is then resuscitated in hospital with supranormal levels of oxygen. Control conditions were 21% Oxygen for 24 hours and addition of LPS to normoxic cells for 24 hours acted as a maximal stimulus.

The results of paired experiments are shown in Graphs 4-10 and 4-11.

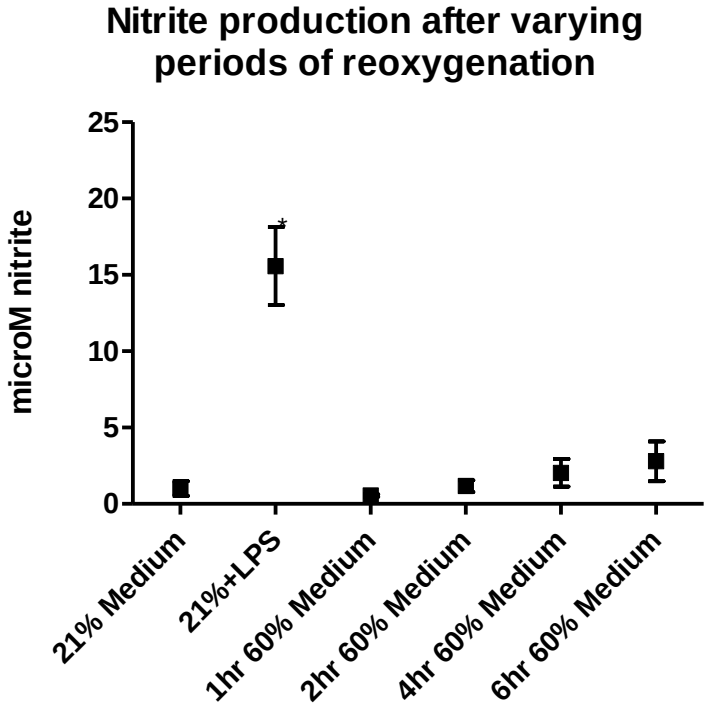


Figure 4-10 Generation of nitrite by MH-S cells exposed to 21% oxygen for 24 hours or 18 hours of 0.5% oxygen followed by varying periods of 60% oxygen. Error bars represent Standard Error. Result of 3 separate experiments with triplicate wells. * p<0.0001 compared to all conditions.

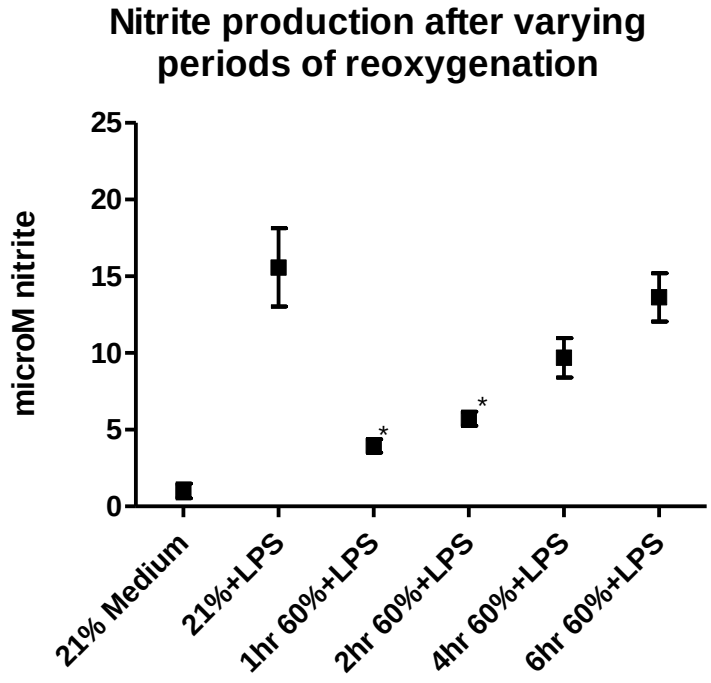


Figure 4-11 Generation of nitrite by MH-S cells exposed to 21% oxygen for 24 hours or 18 hours of 0.5% oxygen followed by varying periods of 60% oxygen in response to LPS. Result of 3 separate experiments with triplicate wells. *p=0.001 compared to 21% oxygen plus LPS.

It can be seen that cells not exposed to LPS again produced minimal levels of nitrite which was not different to cells grown at 21% for 24 hours. Reoxygenation over a 6 hour period however resulted in a rapid increase in nitrite in the medium with levels by 4 and 6 hours not being significantly different from 24 hour 21%+LPS levels. This suggests that the lack of NO in cells exposed to LPS and grown at 0.5% oxygen may be due to substrate limitation (i.e. nitric oxide synthases are present but unable to generate NO as no Oxygen is available to undertake the reaction).

4.4.4 Inducible Nitric Oxide Synthase expression in MH-S cells

To further investigate the production of NO by MH-S cells in response to LPS in hypoxia the expression of inducible Nitric Oxide Synthase (iNOS) was studied by Western blot. Cells were cultured for 24 hours at room air or in 0.5% oxygen in the presence or absence of LPS. A representative immunoblot is shown below:

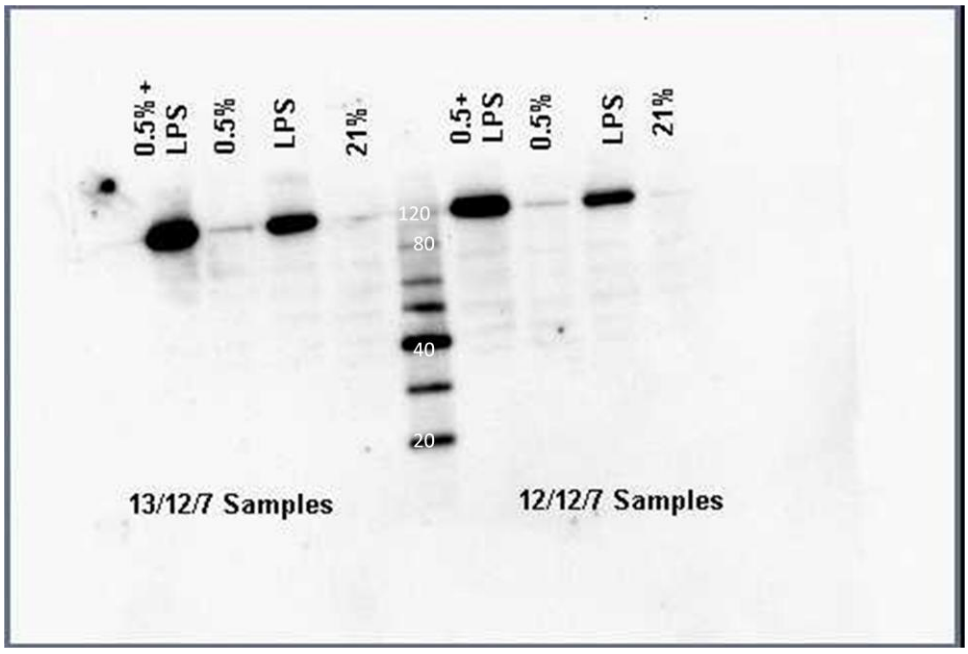


Figure 4-12 Western blot showing increased expression of inducible Nitric oxide synthase in the presence of LPS in 21% or 0.5% oxygen Results from 2 separate experiments shown. Numbers indicate molecular weight markers.

iNOS is detected as a band at 130kDa. As expected there is minimal expression at 21% oxygen in the absence of LPS. LPS results in a large increase in protein expression after 24 hours culture. Hypoxic culture alone results in a small increase in iNOS protein consistent with the reports that iNOS has an HRE as part of its promoter sequence. There is a further increase in iNOS protein in cells grown in 0.5%oxygen

and LPS. This supports the suggestion that in severe hypoxia NO generation is limited by the availability of one of the main substrates, oxygen.

4.4.5 Effect of the Prolyl hydroxylase inhibitor, Dimethyloxalylglycine, on MH-S cells

Some of the effects of hypoxia on the response to LPS in MH-S cells may be mediated by an increase in Hif-1α protein as a result of lack of hydroxylation of Hif-1α and hence its degradation. To investigate this, cells were cultured with DMOG, LPS or both. (Figure 4-13).

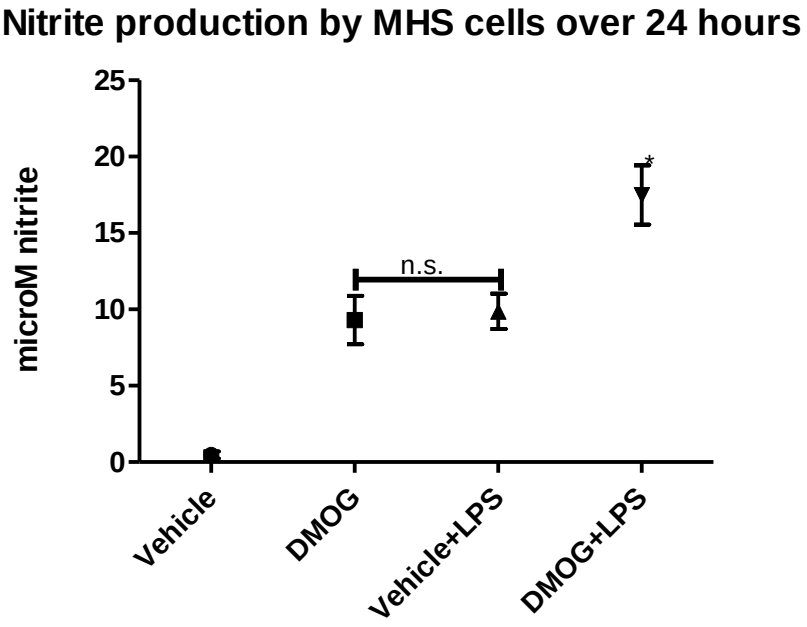


Figure 4-13 generation of nitrite by MH-S cells in the presence of DMOG (1mM) and LPS. Error bars represent Standard Error. Result of 3 separate experiments with triplicate wells. *p=0.001 compared to vehicle, DMOG or LPS. n.s.=no significant difference.

As predicted LPS resulted in increased levels of nitrite compared to medium plus vehicle alone. DMOG however also increased the level of nitrite to a similar level as LPS while the combination of DMOG plus LPS gave a further significant increase in nitrite levels in medium over 24 hours.

The production of TNF in these experiments was also measured. (Figure 4-14).

The addition of DMOG to resting cells had no effect on the production of TNF. However, in the presence of LPS DMOG resulted in a significant decrease in the release of TNF into the medium.

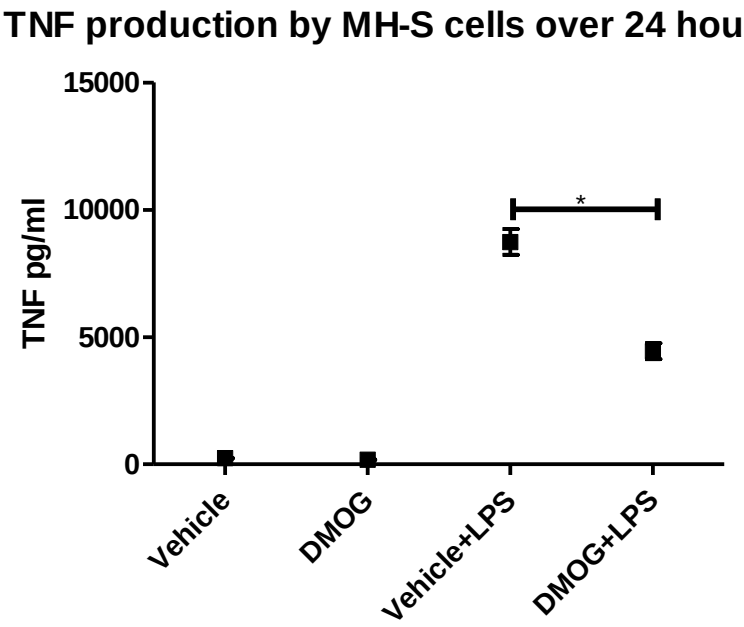


Figure 4-14 Release of TNF by MH-S cells in the presence of DMOG (1mM) and LPS. Mean result shown. Error bars represent Standard Error. Result of 3 separate experiments with triplicate wells. *=p<0.0001. Both LPS treatments were significantly greater than corresponding control p<0.0001.

To see if this was a specific effect for TNF or the result of a general decrease in cytokine secretion the levels of MIP-2, an important chemokine in the recruitment of neutrophils in ALI in mice [204], were measured by ELISA (Graph 13).

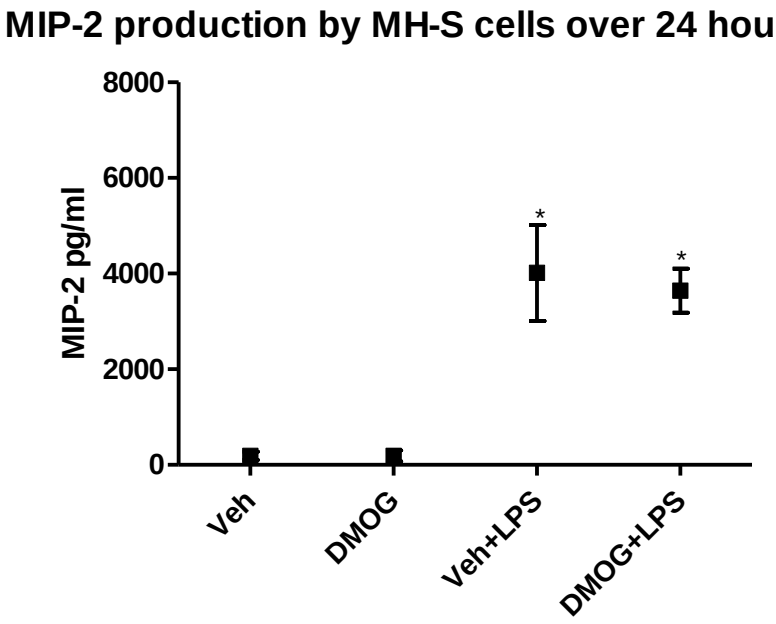


Figure 4-15 Release of MIP-2 by MH-S cells in the presence of DMOG (1mM) and LPS. Mean of three separate experiments with triplicate wells. Error bars represent Standard Error. *p<0.05 compared to control condition.

There was no effect of DMOG on the LPS induced release of MIP-2 in these experiments suggesting a specific effect on TNF synthesis or release.

4.4.6 MH-S cells do not express CXCL-12

It was of interest to define the ability of our macrophage line to express CXCl-12 as it is the first Hif-1 regulated chemokine to be described [205] and its expression has been described in human macrophages.

No CXCL-12 was detectable in the supernatant of MH-S cells cultured for 24 hours in the presence or absence of LPS. This finding was confirmed by analysis of mRNA expression by real time reverse transcription PCR which failed to detect any CXCL-12 transcription in control cells or cells treated with LPS. It would therefore seem that MH-s cells do not express CXCL-12.

4.4.7 DMOG up-regulates several NOS isoforms in MH-S cells.

The level of iNOS after addition of DMOG was analysed by Western blot (figure 4-16). These showed that DMOG resulted in a small increase in iNOS expression after 24 hours culture with DMOG but less than is seen with LPS. The combination of DMOG+LPS results in a greater synthesis of iNOS than LPS alone.

Alveolar macrophages have been reported to express other isoforms of NOS[206] so the expression of endothelial NOS (eNOS) (figure 4-17) and neuronal NOS (nNOS) (figure 4-18) were investigated. DMOG consistently resulted in an increase in the level of eNOS in MH-S cells. There was constitutive expression of eNOS in MH-S cells consistent with the report of Miles et al[206] and LPS had little effect on its expression. DMOG+LPS expressed more eNOS than LPS alone. Finally the expression of nNOS was studied. This was done twice but the results were not consistent. A band at 155kDa could be detected in resting cells. One blot showed an increase in nNOS with the combination of DMOG+LPS while the other showed no significant change in expression due to less protein loading in the treatment lanes.

This is the first study to look at the expression of all 3 isoforms of NOS in an alveolar macrophage line and shows that all eNOS and nNOS are expressed constitutively and that both eNOS and iNOS are inducible in response to LPS or DMOG. It would seem that the increase in nitrite levels detected after the coculture of cells with DMOG and LPS can be explained by an increase in both iNOS and eNOS levels and possibly also by nNOS.

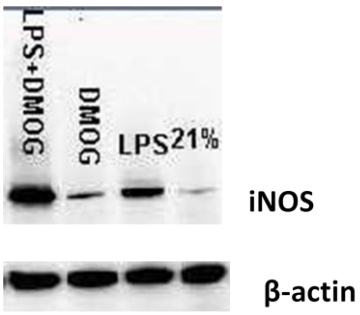


Figure 4-16 Inducible Nitric oxide Synthase expression in MH-S cells exposed to Room air (21%), LPS (1mg), DMOG (1mM) or LPS+DMOG for 24 hours. β-actin control confirms equal loading. Representative blot of 3 separate experiments.

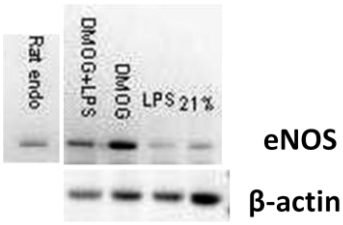


Figure 4-17 Endothelial Nitric Oxide Synthase expression in MH-S cells exposed to Room air (21%), LPS (1mg), DMOG (1mM) or LPS+DMOG for 24 hours. β-actin control confirms equal loading. Representative blot of 3 separate experiments. Rat endothelial extract was run as a positive control.

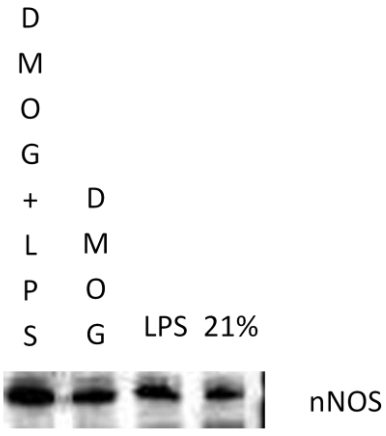


Figure 4-18 Neuronal Nitric Oxide Synthase expression in MH-S cells exposed to Room air (21%), LPS (1mg), DMOG (1mM) or LPS+DMOG for 24 hours. One of 2 experiments which showed inconsistent results.

4.4.8 Up regulation of HO-1 protein by LPS+DMOG

The down regulation of TNF expression by the combination of LPS+DMOG was unexpected following the finding of no effect of hypoxic culture+LPS in MH-S cells. One possible explanation for the finding is that DMOG+LPS were a stronger inducer of HO-1 expression in these cells. HO-1 has an HRE and has been shown to have anti-inflammatory effects in macrophages on macrophages acting by the generation of carbon monoxide[207]. To investigate this possibility the expression of HO-1 protein was studied by western blot (figure 4-19).

MH-S cells constitutively expressed HO-1 and there was significant increase in the band density when cells were cultured with DMOG or concurrent LPS+DMOG compared to control, LPS alone or 0.5% oxygen. This raises the possibility that at least some of the reduction in TNF expression in these cells is due to the increased expression of HO-1.

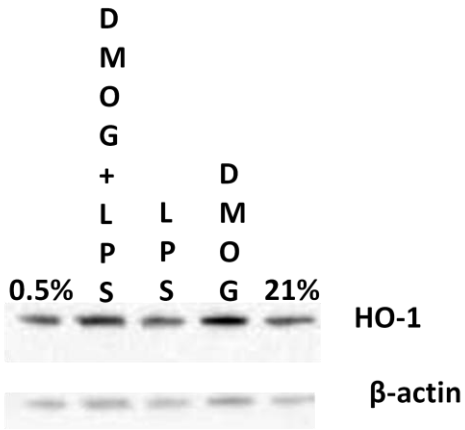


Figure 4-19 Expression of HO-1 in MH-S cells exposed to Room air (21%), DMOG (1mM), LPS (1µg) both DMOG+LPS or hypoxia (0.5% Oxygen). β-actin control confirms equal loading. Representative of 3 separate experiments.

4.4.9 Expression of Hif-1α protein under varied culture conditions

All the variables manipulated in the above series of experiments may have at least some of their effects through the alteration (increase) of Hif-1α protein. This may occur either by increased protein stability (hypoxia, DMOG) as a result of inhibition of the PHD enzymes or increased transcription and translation[142;208].

Unfortunately the detection of Hif-1α protein proved to be more problematic than anticipated in our murine cell line. Throughout nuclear protein was isolated for analysis. Previous success had been achieved with human cells in the CIR[209] using a mouse anti-human monoclonal antibody to Hif-1α. In my hands under a range of conditions I could detect no protein expression using this antibody.

Subsequently the ab-1 antibody from Abcam was tried. This resulted in a faint band in hypoxic cells at about 95kDa. Discussion with Dr Thorsten Crammer revealed that his group had had similar difficulties in detecting Hif-1α protein by Western blot and had recommended a polyclonal rabbit anti-human antibody (Cayman Chemicals Cat No.10006421).

A western blot comparing the 3 described antibodies is shown below. The BD antibody revealed no detectable band, both ab-1 and the Cayman antibody showed a band at 95kDa thought to be Hif-1α. The Cayman ab gave the strongest signal despite there being least protein in those lanes and so it was subsequently used for analysis.

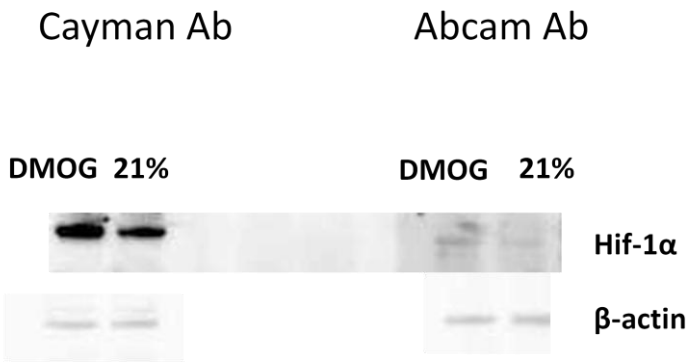


Figure 4-20 Western blot showing variable ability of Hif-1 antibodies to detect protein. Control cells were cultured in room air for 24 hours. DMOG treated cells cultured in the presence of 1mM for 24hours.

To confirm that the 95kDa band was Hif-1α a western blot was performed (figure 4-20) on nuclear protein obtained from bone marrow derived macrophages obtained from Cre Negative (i.e. wild type) and Cre Positive (i.e. Hif-1α deplete) Hif-1 Cre/lox mice cultured under 0.5% oxygen + DMOG to maximally induce Hif-1α. Nuclear protein from Cre negative (wild type) and Cre positive (Hif-1 over-expressing) VHL bone marrow macrophages cultured at 21% oxygen were also analysed. This immunoblot confirms that the Cayman antibody detects Hif-1α as a protein at about 95kDa (detectable band in WT hypoxic Hif mice and absent in Hif deplete cells; detectable band in Hif over-expressing cells at 21% while minimal in wild type).

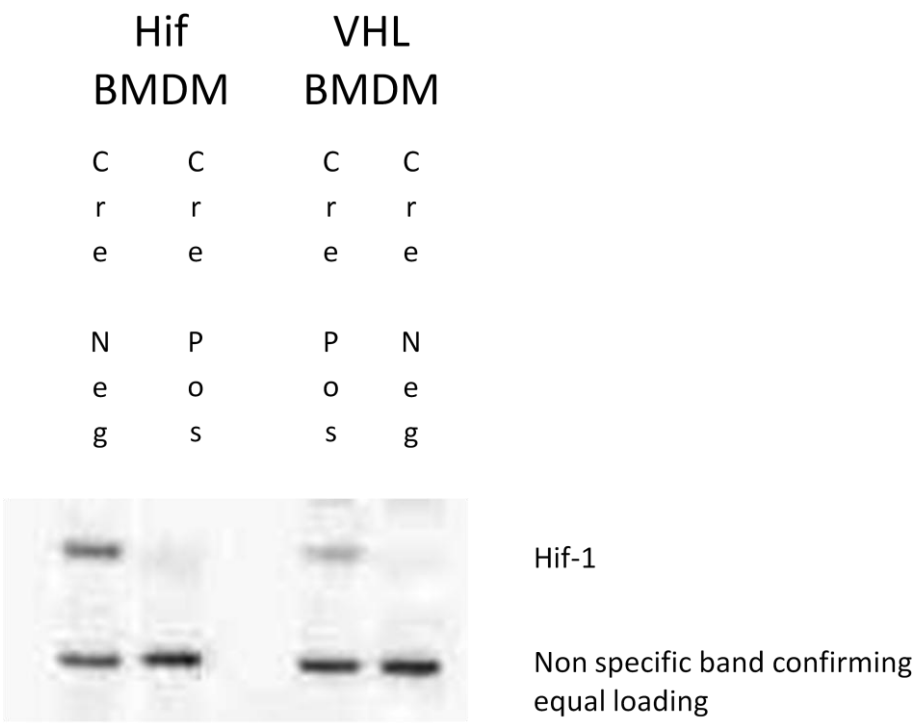


Figure 4-21 Western blot using Cayman Antibody to Hif-1 in BMDM from Hif Cre Negative and Positive cultured in hypoxia and DMOG (to maximise Hif-1 stabilisation) and VHL Cre Negative and Positive animals cultured at 21% (to increase Hif destruction).

Subsequently it could be shown that culture with DMOG, LPS, DMOG+LPS and 0.5%oxygen for 24 hours increased the nuclear levels of Hif-1α in a single experiment. Cells exposed to hypoxia seemed to have the greatest increase with DMOG exposed cells having the next largest increase.

4.4.10 NF-κB activity in MH-S cells

In light of the unexpected finding of reduced TNFα production in the presence of DMOG the levels of another transcription factor, NF-κB, known to be important in the regulation of TNFα production was studied.

Nuclear protein was collected at 2 and 24 hours and the p65 level measured by Active Motif Trans AM assay

Veh 24 hr	222.73
DMOG 24Hr	209.12
Veh + LPS 2 Hr	918.20
DMOG +LPS 2 Hr	871.46
Veh 24 hr	302.45
DMOG 2Hr	203.05
Veh + LPS 24 Hr	710.68
DMOG +LPS 24 Hr	643.08

It can be seen that both LPS and LPS+DMOG increase p65 levels at both 2 and 24 ours post-stimulus. DMOG alone appears in this cell type to have no effect on p65 level at 2 or 24 hours.

4.5 Discussion

This chapter has been concerned with the response of the MH-S cell line, a well characterised murine alveolar macrophage line derived by SV40 viral transformation of primary alveolar macrophages obtained from Balb/c mice [143;144] to LPS and the manipulation of this response.

The initial findings of a dose responsive, prolonged generation of NO measured by supernatant levels of nitrite are consistent in both quality and quantity with that of previous researchers [203] who have employed this cell line. Alveolar macrophages are known to be an important source of NO in ALI hence the results of this chapter should be of relevance to in vivo events.

4.5.1 Effect of varying ambient oxygen

Murine macrophages secrete NO in response to LPS alone, unlike human macrophages which need a cocktail of stimuli to induce NO production [210]. There are three isoforms of NOS; Type I (neuronal); Type II (inducible) and Type III (endothelial). Types I and III are constitutively expressed, calcium dependent and secrete low levels of NO. Inducible nitric oxide Synthase (iNOS) is a calcium independent, high output enzyme which continues to produce NO until the enzyme is degraded. Both iNOS [211] and eNOS [212] have been shown to possess an Hypoxia-response element in their promoter sequence and nNOS has also been shown to be regulated by hypoxia[213]. Alveolar macrophages are an important source of NO in ALI and thus the potential effects of varied alveolar oxygen as a result of the initial insult or subsequent resuscitation need to be understood.

Initially three levels of ambient oxygen were studied, 21% (room air), 14% and 0.5%. 14% was chosen to approximate to normal alveolar pO_2 and comparison with room air showed no significant difference in NO generation, consistent with the findings of others [214]. This allowed us to remove the 14% group from future studies for ease of experimental design.

A major finding was that severe hypoxia reduced the generation of NO in the supernatant in response to LPS despite an increased expression of iNOS. This is in agreement with other studies using macrophage cell lines derived from other primary cells (ANA-1, a C57/Bl6 bone marrow derived macrophage cell line transformed by the J2 retrovirus, [211]; Raw 264.7, derived from Balb/c peritoneal macrophages transformed by Abelson virus [214;215]. The ability of reoxygenation to rapidly increase the supernatant levels of NO suggests that oxygen availability is the rate limiting step in the generation of NO in severe hypoxia. This increase in NO generation upon reoxygenation has not been shown by previous investigators[202] although they used 21% oxygen to reoxygenate and a shorter (2 hour) period, suggesting that more rapid delivery of oxygen results in more rapid restoration of NO generation. Furthermore, it has been shown in humans in vivo that the rate limiting step for the production of NO in lungs is inspired oxygen[216] and that major reductions in exhaled NO are not measurable until inspired oxygen is as low as 5%.

Prolonged hyperoxia had no effect compared to room air on the amount of NO generated.

In agreement with others[211] hypoxia alone had little effect on iNOS protein levels, despite the presence of an HRE in the iNOS promoter. Instead, hypoxia seems to act as a co-stimulus to increase the levels of iNOS synthesised. This may be a control

mechanism to prevent inappropriate synthesis of iNOS in the absence of other inflammatory stimuli.

As expected, LPS resulted in the production of large quantities of TNF by MH-S cells. The effect of varying oxygen on TNF production following LPS stimulation in the literature is conflicting. Using primary alveolar macrophages from rats Leeper-Woodford & Detmer [83] found that 1.8% oxygen increased the LPS generated release of TNF. VanOtteren et al [82] studying the response of murine primary alveolar macrophages suggested that 12 hours of anoxic culture resulted in a small statistically not significant increase in TNF mRNA and that 6 hours of anoxia followed by 12 hours reoxygenation resulted in a significant increase in TNF protein secretion. Others, have found no effect on TNF secretion using BMDM derived[200]) or peritoneal derived [201;215] cell lines. Hence the finding in this system that varying ambient oxygen does not affect TNF secretion is in keeping with the results from other cell lines.

4.5.2 Effect of DMOG

DMOG, a prolyl hydroxylase inhibitor, has been shown to increase the expression of hypoxia regulated genes in a range of cell types. Its mode of action is thought to be through inhibition of the prolyl-hydroxylases, PHD 1-3, leading to stabilisation of Hif-1 (see figure 4-22) although other mechanisms have very recently been proposed [217].

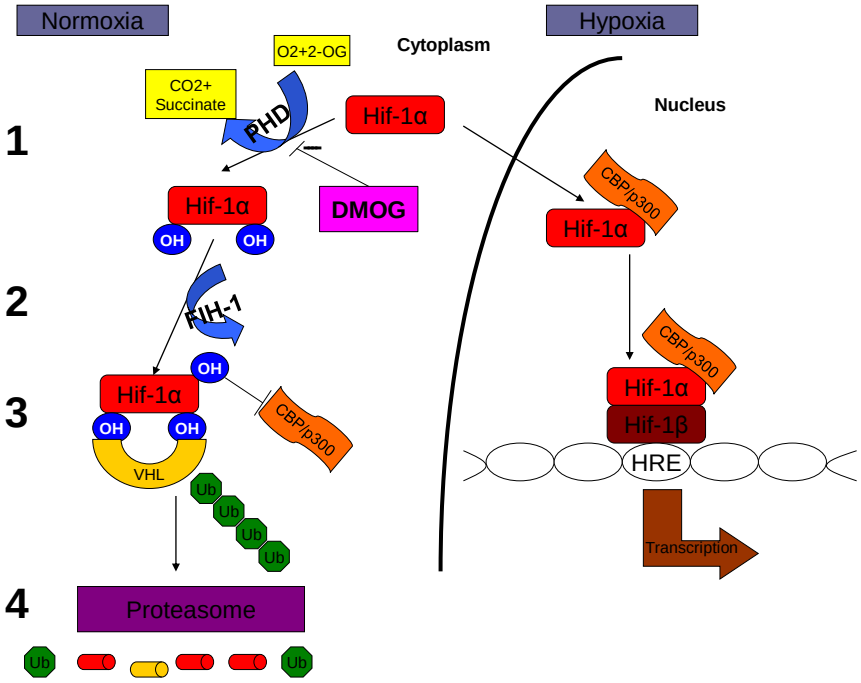


Figure 4-22 Mechanism of action of DMOG. The main action is thought to be the inhibition of PHD activity.

This is the first study we are aware of where the effect of DMOG on alveolar macrophage responses to inflammation has been studied.

DMOG increases macrophage generation of NO alone and has an additive effect with LPS. Alone, DMOG caused an increase in iNOS protein (although less than LPS

alone) and the combination of DMOG and LPS caused a greater expression of iNOS than LPS. Hence the additive effect of DMOG and LPS can be explained by a further increase in NO generation as a result of increased enzyme levels. However, the generation of similar amounts on NO by DMOG alone compared to LPS seems harder to explain as, despite an increase in iNOS expression with DMOG the increase is less than LPS alone.

This led to the study of the expression of other isoforms of NOS in the MH-S line. We have shown for the first time that MH-S cells express both eNOS and nNOS. eNOS is known to have an HRE and we have shown that DMOG alone results in an increase in eNOS expression. Whether the observed generation of NO by DMOG treated MH-S cells to levels similar to that occurring in response to LPS is due to the combined increase in iNOS (high output enzyme) and eNOS (low output enzyme) in DMOG treated cells is uncertain and deserves further study. A possible role for nNOS in the generation of NO in response to DMOG could not be confirmed because of conflicting results however, nNOS has been shown to be regulated by hypoxia and its promoter has potential Hif-1 binding sites[213].

A consistent finding was that the generation of TNF in response to LPS was significantly reduced by DMOG exposure. This was an unexpected finding in light of Peyssonnaux et al's [218] finding that Hif-1 deplete BMDM generate reduced TNF in response to Group A streptococci. However our results are in agreement with recent in vivo findings that DMOG reduces TNF release in inflammation [217;219]. In our system this would seem to be a specific effect on TNF rather than a non-specific inhibition of cytokine release as the release of MIP-2, a potent neutrophil chemokine that is the murine equivalent of Il-8 and has been shown to be important in the

development of ALI in mice [74;204] was unaffected. The mechanism for DMOG's reduction in TNF release is unclear. A possible Hif-1 mediated pathway for the reduction in TNF release is through induction of HO-1 by DMOG. HO-1 is the rate limiting step in the oxidative degradation of heme to CO, iron and bilirubin. HO-1's products have been shown to have anti-inflammatory properties [207;220;221]. Using western blot we have shown that MH-S cells express HO-1 and that the combination of DMOG and LPS increase the expression of the enzyme to greater levels than DMOG or LPS alone. Hence the reduction in TNF release may be mediated by HO-1 products such as CO acting via a cGMP dependent pathway. The availability of HO inhibitors such as mesoporphyrin and HO knockout mice would allow further investigation of the possible mechanisms involved.

An alternative explanation for the alteration in supernatant TNFα levels would be that DMOG induces a change in the proportion of membrane bound to soluble TNFα [222]. TNFα is produced initially as a 26kDa membrane associated form (mTNFα) which is then released as the soluble 17kDa (solTNFα) form by the action of TNFα-converting enzyme (TACE) [223]. It is known that there is increased expression of mTNFα in patients with ARDS [222] and that while solTNFα levels may reflect systemic TNF activity it is thought that mTNFα levels may be a better reflection of the pulmonary TNF activity. Alveolar macrophages expressing mTNFα may have a direct interaction with the alveolar-capillary barrier leading to DAD. Alternatively the action of TACE could be affected by the DMOG. LPS is known to increase the levels of TACE in alveolar macrophages [224] and DMOG may have an unexpected effect on the synthesis or activity of this enzyme. TNFα ELISAs are thought to recognise the solTNFα but can also recognise the receptor bound form [225] which may have biological action and can interfere with the recognition of solTNFα. The

suppliers of the TNFα ELISA used in this body of work are unable to identify the epitope that the capture antibody recognises on the TNFα molecule. Future work should study the effects of DMOG on the levels of mTNFα, the release of TNF receptor and the activity of TACE in the presence of LPS.

4.5.3 Expression of Transcription factors in MH-S cells

In this study the measurement of 2 transcription factors was attempted. Determining the expression of Hif-1 by MH-S cells proved more difficult than expected from review of the literature. Using a rabbit polyclonal antibody we were able to show the presence of an oxygen sensitive band at 95kDa. The identity of this band was confirmed using BMDM from Hif-1 deleted cells exposed to hypoxia which showed reduced staining compared to wild type and normoxic expression of the protein by VHL (Hif-1 over-expressing) cells in normoxia. The literature frequently describes Hif-1 on western blot as a 120kDa band but these reports are usually from human studies [226;227]. Other studies have shown that murine Hif-1[228] can be detected at around 95kDa. In agreement with others work [142;208] LPS resulted in an increase in Hif-1 protein at 24 hours. Unfortunately it was not possible to further study the kinetics of Hif-1 expression in this model due to time constraints.

In light of a report that DMOG may affect the activity of components of the NF-κB pathway[229] a single experiment was made to study p65, a component of NF-κB expression. As expected LPS increased p65 levels but interesting DMOG appeared to have no effect in this cell line. However this limited study could not determine any effect of DMOG on p65 levels. This needs repeated. The effect of DMOG on NF-κB in alveolar macrophages may differ to that shown in intestinal epithelial cells[217] but the importance of IκB kinase in macrophage activity [230-232] and its inhibition by PHDs[229] means that further study is required.

4.6 Conclusion and further work

This chapter has been concerned with the response of an alveolar macrophage cell line to stimuli relevant to lung injury (LPS and hypoxia/hyperoxia) and the effect of a pharmacological agent (DMOG) on some of these responses.

This study has shown that MH-S cells express all the isoforms of NO Synthase and that they have differing responses to LPS. It has also confirmed that ambient oxygen is a rate determining feature of NO synthesis by alveolar macrophages.

Furthermore it has shown that DMOG increases the expression of NO (an anti-bacterial agent) while reducing the secretion of TNF, an important pro-inflammatory cytokine. This raises the possibility that administration of DMOG may enhance bacterial killing via NO generation while reducing other potentially deleterious features of the inflammatory response induced by TNF. This will be examined further in chapter 6.

In order to clarify the role of the different isoforms of NO Synthase on NO generation in response to DMOG the use of BMDM from knockout mice would be invaluable. Unfortunately they were not available to us during this project but the response of nNOS, iNOS and eNOS knockout cells to DMOG would be interesting. Furthermore the use of HO-1 knockout cells to determine the relevance of the observed increase in HO-1 expression in response to DMOG and its potential effect on LPS induced TNF generation would be important. As already discussed the measurement of mTNFα and TACE activity would help clarify the mechanism for the reduction in TNFα levels in medium following LPS exposure.

Now that the tools are available to study the levels of Hif-1 protein in MH-S cells it would be valuable to study these in more detail over varying durations and doses of LPS exposure. As discussed above the role of the NF-κB pathway in mediating the effects of DMOG requires investigation. The availability of techniques such as short-interfering RNA would allow the exploration of the importance of Hif-1α and NF-κB mediated effects to be explored by using DMOG to increase the level of the transcription factor of interest while blocking the activity of the other. The interaction between DMOG and Hif should also be explored using the availability of Hif-1α [89] (see chapter 5) and more recently Hif-2α [233] myeloid knockout mice.

Chapter 5

Endotoxin induced lung injury in Myeloid cell Hif-1α deficient mice

5.1 Introduction

The use of genetically modified animals, particularly mice, has proven to be a powerful tool in biological science allowing researchers to understand the role of specific molecules in the generation of biological responses *in vivo*. It is possible to alter the mouse genome with nucleotide precision allowing the study of single gene function in intact animals and in models of human health and disease.

Hif-1α is an important gene in embryonic development. A Hif-1α knockout mouse has been generated but it is a lethal mutation with embryonic death occurring by day E11 as a result of severe cardiovascular and neural tube defects [94] . This has lead to other approaches to study the effect of Hif-1α expression in adult animals. Using the Cre/lox system [234] Randal Johnson's group at the University of California have been able to generate mice with a targeted deletion of Hif-1α in myeloid cells [89]. They have previously reported the generation of a loxP-flanked (floxed) allele of Hif-1α in mouse embryonic fibroblasts [103] which in the presence of cre recombinase results in the excision of a segment of exon 2 which encodes the helix-loop-helix motif which is essential for Hif dimerisation with Hif-1β and subsequent transcriptional activity. Targeted deletions of Hif-1α mice were created by crossing this floxed allele into the LysMCre mouse [235]. The LysMCre mouse expresses the enzyme Cre recombinase under the control of the Lysozyme M promoter. This results in the expression of Cre in all myeloid cells. This cre expression then results in the excision of the floxed segment of Hif-1α and the generation of myeloid cells which are unable to generate functional Hif-1α (Fig 5-1). This animal is therefore a powerful tool with which to explore the role of Hif-1α in the generation of inflammatory injury.

Professor Johnson supplied our group with breeding pairs of the Hif-1α knockout mouse with which we were able to generate our own breeding colony.

The hypothesis tested in this chapter was that Hif-1α myeloid cell knockout mice would have attenuated lung injury in response to endotoxin.

The aims of this chapter were to:

-confirm the genotype of the breeding colony

-establish the expression of cre in alveolar macrophages

-confirm the action of cre in alveolar macrophages and establish the deletion efficiency of the system

-study the role of myeloid cell Hif-1α in response to intratracheal LPS

-confirm the key role of Hif-1α in generating NO in response to LPS

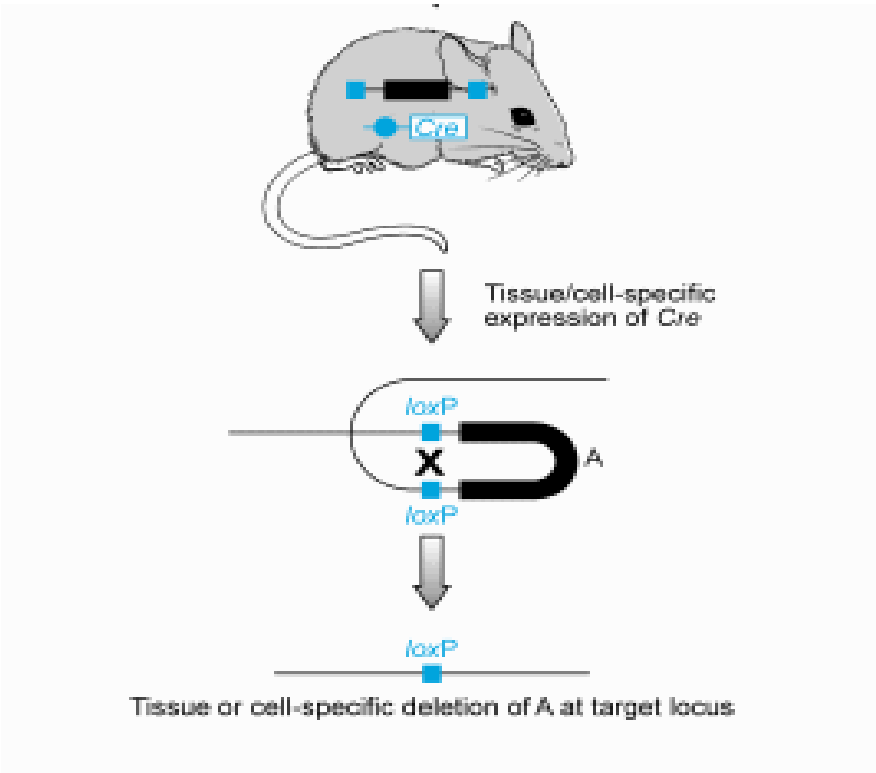


Figure 5-1Summary of the mechanism of the cre/lox system. The gene of interest is flanked on both sides "floxed" by the bacteriophage sequence loxP (locus of X-over P1). Under the control of a specific promoter element (in this works case Lysozyme M) the enzyme Cre (cyclization recombination) is expressed in the cell of interest (here the myeoloid cell line). The action of cre is to excise the floxed sequence and hence prevent the transcription of functionally active mRNA.

5.2 Results

5.2.1 Confirmation of presence of floxed alleles

The Hif-1α conditional deleted mice (referred to hereon as Hif knockout [KO]) were maintained in the Edinburgh University Biomedical Research Resources animal facility at Little France. Genotyping for cre expression was performed in house by BRR staff by real time PCR with heme oxygenase 1 run as a positive control. Ear clip samples were used to provide DA which was extracted by standard methods.

Using sequences provided by Prof. Johnson the presence of floxed alleles was confirmed by PCR and gel electrophoresis (Fig 5-2)

Figure 5-2 Genomic PCR confirming presence of loxP sites in Hif KO mice. –ve=negative control sample(water) SH29-32 are Hif KO animals, Normal 1 and 2 are wild type mice. The floxed allele is shown at 250bp and wild type at 200bp.

5.2.2 Bone Marrow Derived Macrophages express Cre recombinase

The cre lox system employed depends on the expression of cre at some point of the cell cycle (controlled in this case by the Lysosyme M promoter). In order to support the findings of Cramer et al [89] that bone marrow derived circulating inflammatory cells are Hif-1α knocked depleted in this strain the expression of the cre recombinase protein in Bone Marrow Derived Macrophages was confirmed by Western blot (figure 5-3).

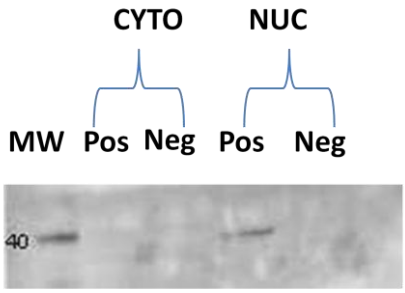


Figure 5-3 Western blot of Cytoplasmic (Cyto) and Nuclear (Nuc) Extracts from Cre Positive (Pos) and Negative (Neg) Hif mice confirming expression of cre in nucleus of PCR typed animals.

5.2.3 Alveolar macrophages express cre recombinase in the Hif mouse.

To confirm the expression of the cre recombinase enzyme in alveolar macrophages of cre positive animals, lung sections from floxed Hif mice which were known by genotyping to be Cre positive and negative were stained for cre by immunohistochemistry. This confirmed the nuclear expression of cre in alveolar macrophages in cre positive animals.

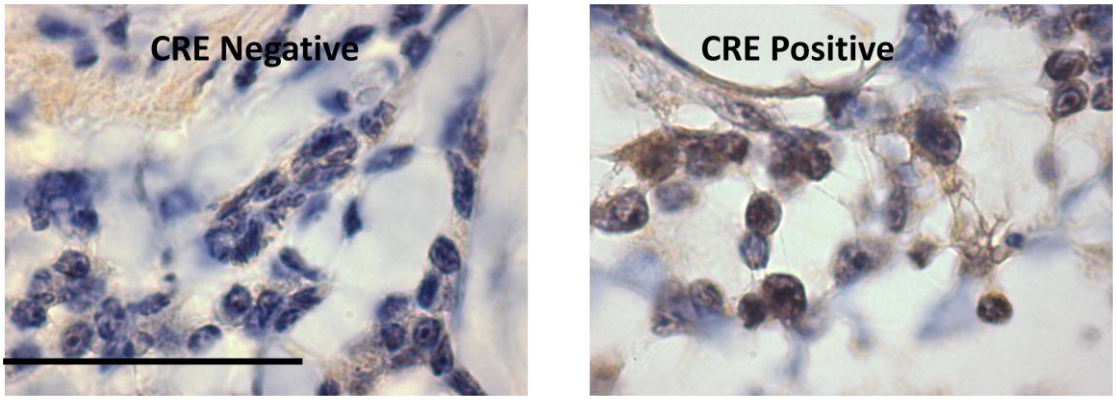


Figure 5-4 Immunohistochemical stain of Lung sections from Cre Negative and Positive animals confirming expression of Cre in alveolar macrophages (x200). Graticule=100µm.

5.2.4 Deletion efficiency of Hif-1α in Mouse Alveolar Macrophages.

Cramer et al [89] have shown that the cre/lox system results in a high degree of deletion of the targeted Hif-1α exon in peritoneal macrophages and neutrophils but the deletion efficiency in other myeloid cells is unknown. Alveolar macrophages were harvested from Cre negative (wild type Hif-1α expression) and Cre positive (KO) animals. Genomic DNA was extracted from these cells and stored. Using quantative PCR the deletion efficiency in alveolar macrophages was shown to be high. HO-1 expression was used as the reference gene against which Hif-1 levels were expressed.

In our hands deletion efficiency in alveolar macrophages is greater than 97% (figure 5-4)

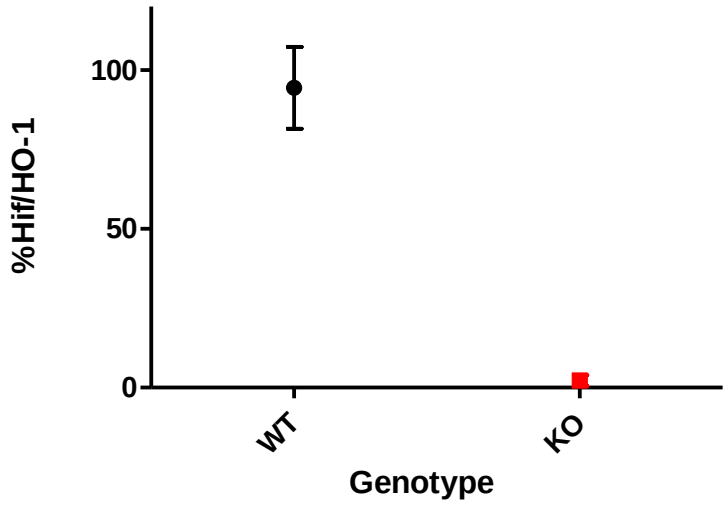


Figure 5-5 Genomic amount of Hif-1 compared to HO-1 in wild type (WT, cre negative) and knockout (KO, cre positive) animals 5 animals per group. Error bar represent SE.

The deletion efficiency in alveolar macrophages is higher than in BMDM and neutrophils as reported by Cramer et al [89]. This likely reflects the nature of the

alveolar macrophage in that it is a mature terminally differentiated cell which has had a prolonged period of growth and differentiation in which cre can be expressed and act on the floxed allele.

5.2.5 Wild type and Hif-1α myeloid knock-out mice have similar alveolar lavage cell populations following intratracheal saline.

A small number of wild type and KO mice were given intratracheal saline and then underwent BAL at 6 hours. The results are shown in Figure 5-5.

Both WT and KO mice had similar macrophage predominant populations with similar BAL protein levels suggesting that the Hif-1α myeloid knockout does not affect resting alveolar populations.

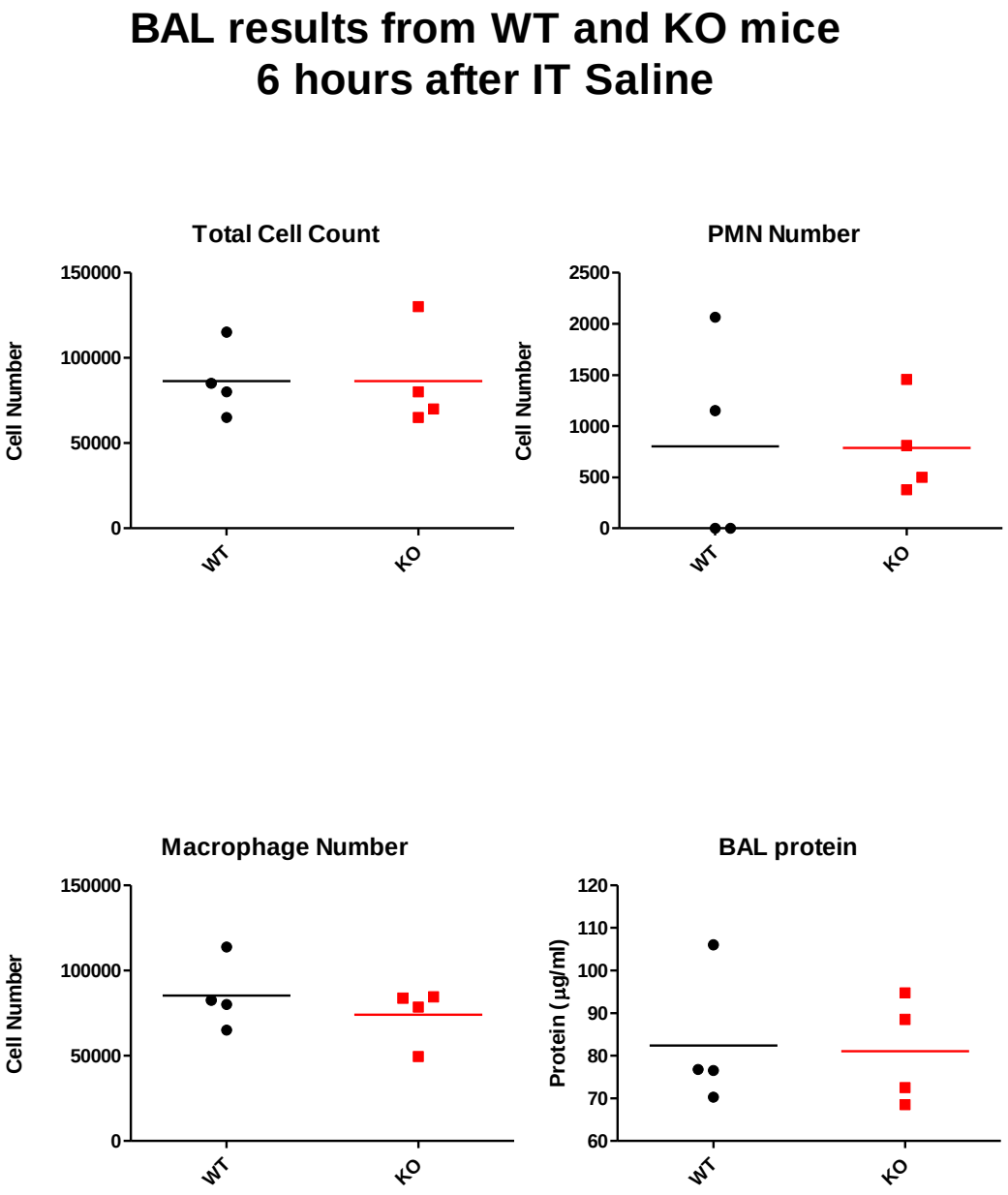


Figure 5-6 Bronchoalveolar lavage results from WT or KO mice given intratracheal saline. Each data point represents an individual animal. Bar equals mean value. No significant differences between wild type and knockout animals by Mann-Whitney test.

5.2.6 Hif-1α myeloid Knock-out mice develop pulmonary inflammation in response to intratracheal LPS.

Subsequent to the above experiment BALs were obtained from 3 groups of KO mice: naïve, unstimulated animals; animals that had received intratracheal saline 6 hours prior to sacrifice and animals that had received intratracheal LPS 6 hours previously.

The cell counts and BAL protein levels are shown in Figure 5-6.

The results confirm that naïve KO mice have an alveolar cell population that is predominantly composed of alveolar macrophages. At the 6 hour timepoint there is also little effect from intratracheal saline. The alveolar population remains predominantly macrophage and there is no evidence of an alteration in alveolar capillary integrity reflected by the lack of a change in BAL protein levels.

Intratracheal LPS however, results in a neutrophilic alveolitis with an increase in total cell number, a sHift to a neutrophil rich lavage population and evidence of alveolar damage reflected by an increase in BAL protein.

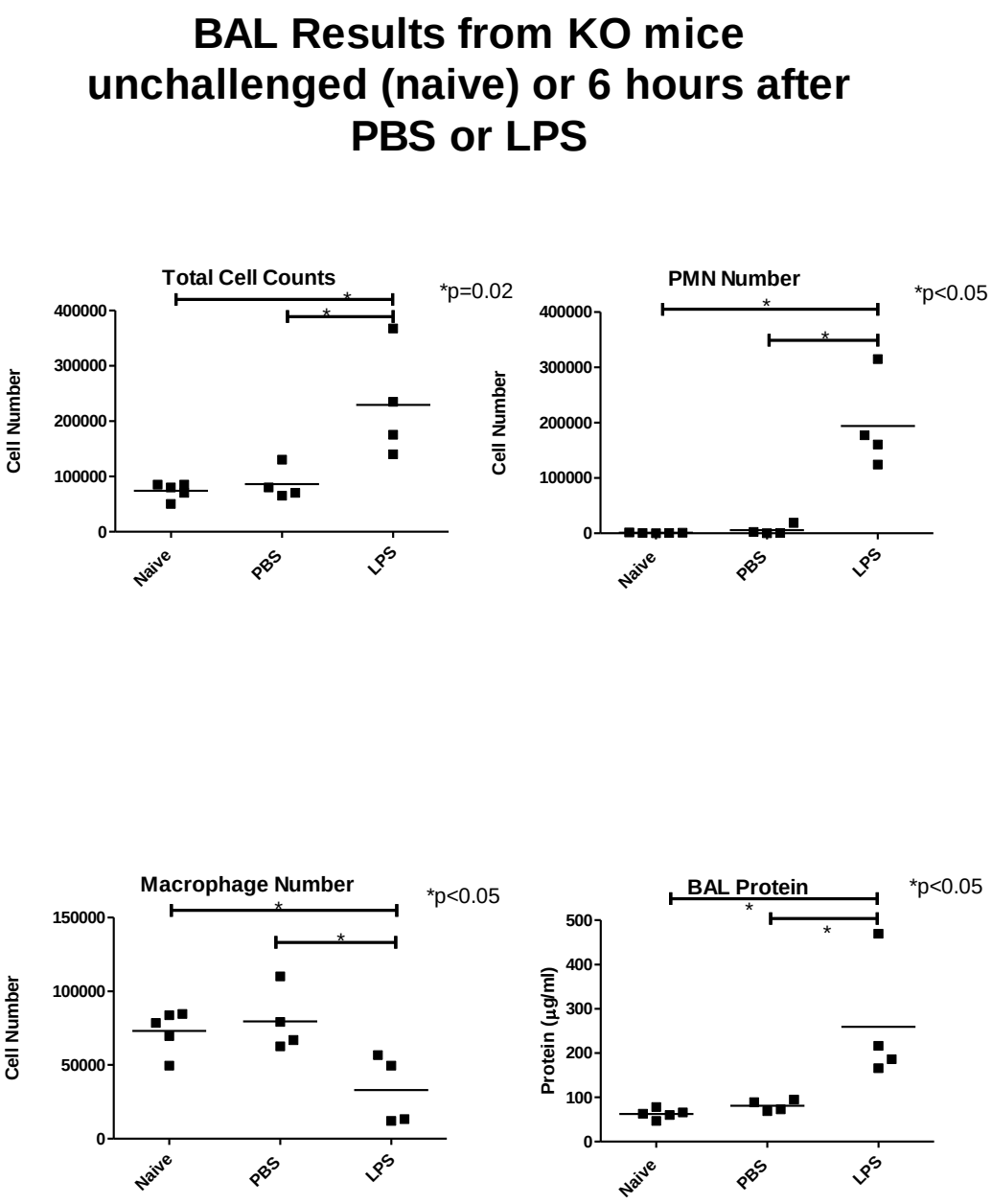


Figure 5-7 Bronchoalveolar lavage results from unchallenged mice or mice given either saline or LPS intratracheally. Each data point represents an individual animal. Bar equals mean value.

5.2.7 Hif-1α myeloid knockout mice develop a similar pattern of Acute Lung injury following intratracheal LPS to wild type mice.

Having shown that KO mice can develop ALI we then compared the response to i.t. LPS at various timepoints. Timepoints ranging from 2 hours to 5 days were studied to allow full characterisation of the response to LPS which has been shown to have differing peaks for inflammatory cell infiltration and alveolar-capillary damage [187;194]. The timepoints chosen were 2 hours; 6 hours; 24 hours; 48 hours and 5 days after i.t. instillation of LPS. The data are summarised in figure 5-7 and the results for each timepoint are shown in more detail in Figs 5-8 to 5-12.

BAL results after intratracheal LPS

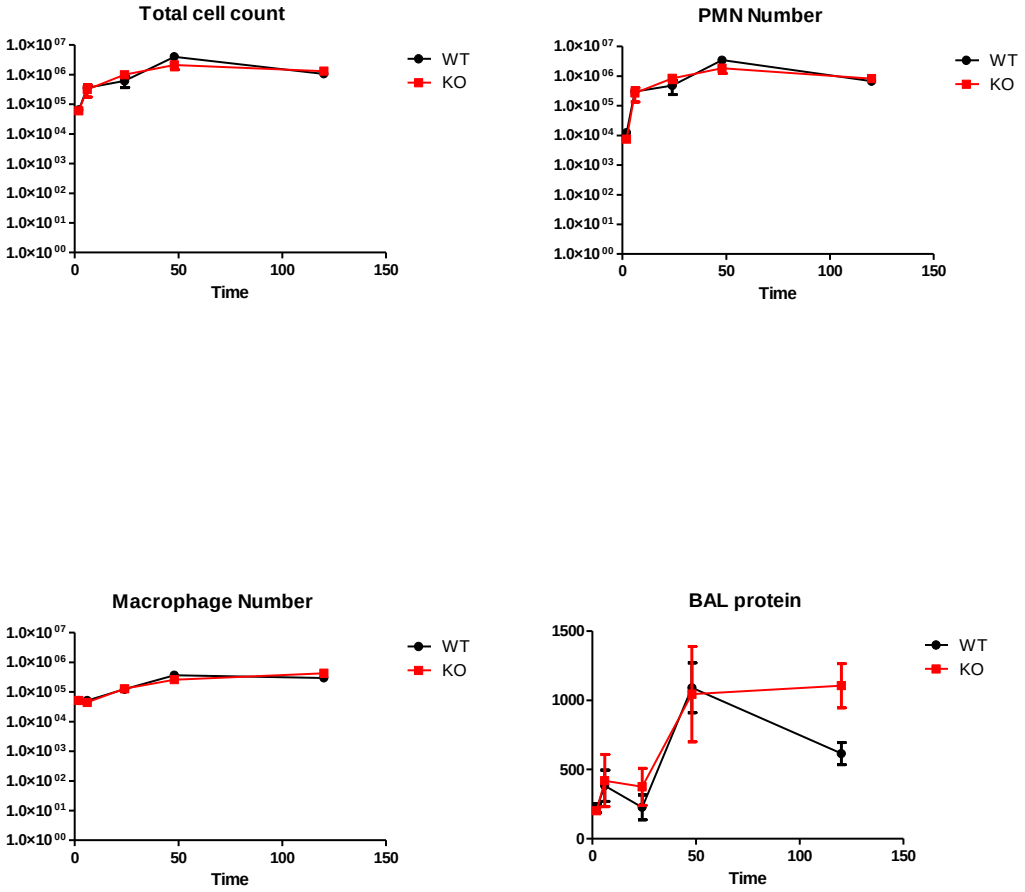


Figure 5-8 Summary data from animals given intratracheal LPS and sacrificed at 2, 6, 24, 48 and 120 hours. Each point represents mean result. Error bars denote standard error. N=3-9 animals per experiment.

Total cell number and neutrophil number are increased by LPS instillation. Both peak at 48 hours after insult. BAL protein also peaks at 48hours following LPS. Comparing WT and KO mice it can be seen that the results are similar, both resulting in an intense neutrophilic alveolitis with an increase in BAL protein representing alveolar-capillary damage.

At 2 hours after LPS instillation there are fewer neutrophils in the KO BAL than WT. 2 hours is the earliest time at which one would expect to see PMN in the BAL as a result of LPS instillation [187] and our findings suggest that KO PMN are slower in their migration to the airspace than WT. This difference however is lost by 6 hours confirming that PMN from KO mice are able to migrate to sites of injury [218]. The ability of these migrated PMNs to cause damage is confirmed by the increase in BAL protein seen during the course of the study.

An interesting observation is that KO BAL protein remains elevated at 5 days following the insult while the WT BAL protein level has dropped significantly (48 hours 1090 ± 180.3 $N=4$ v 120 hours 615.6 ± 79.51 $N=7$, $p<0.05$).

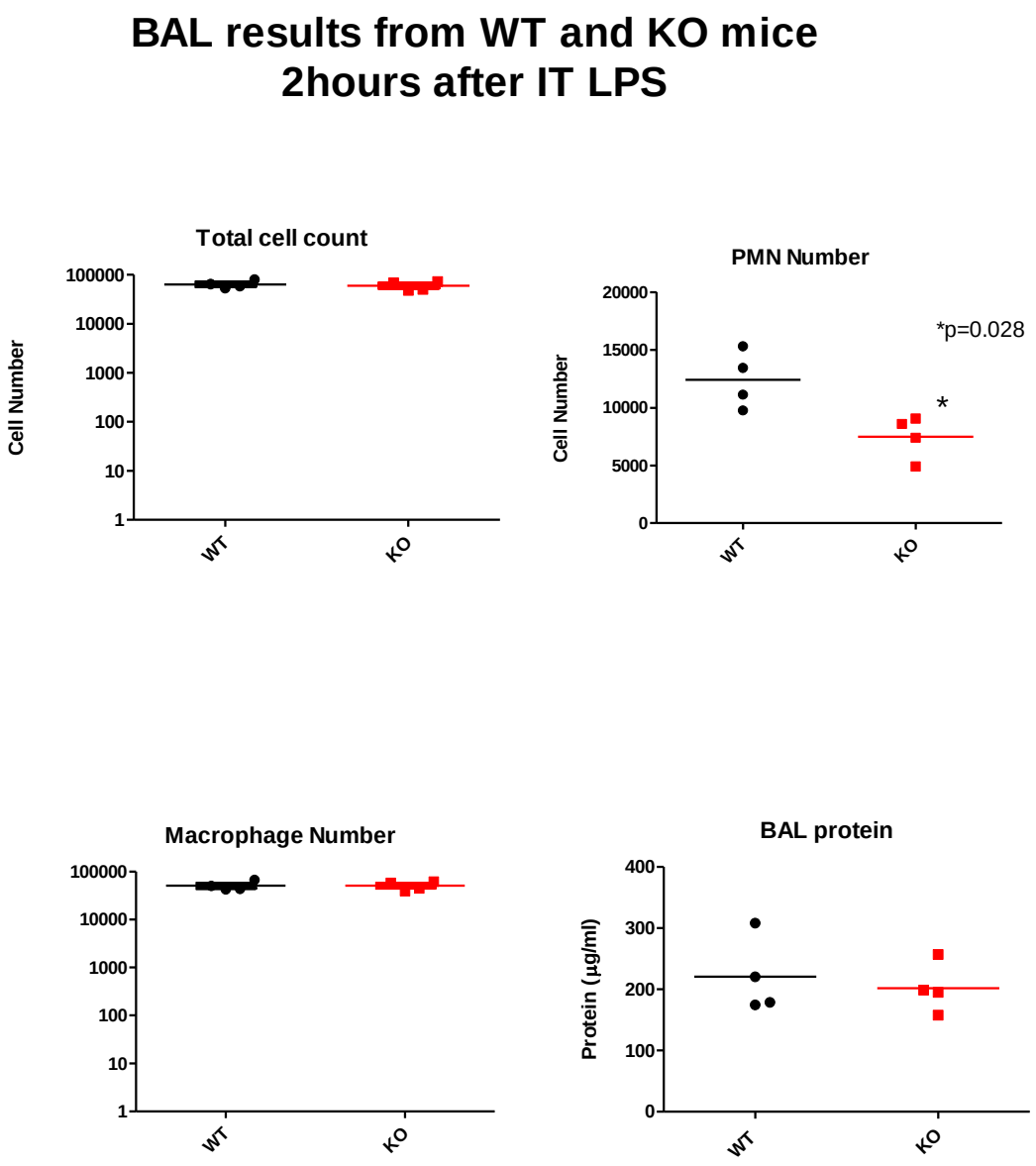


Figure 5-9 Bronchoalveolar lavage results from WT and KO animals given 50 µg intratracheal LPS and sacrificed at 2 hours post-instillation. Each data point represents an individual animal. Bar equals mean value. Groups compared by Mann-Whitney test.

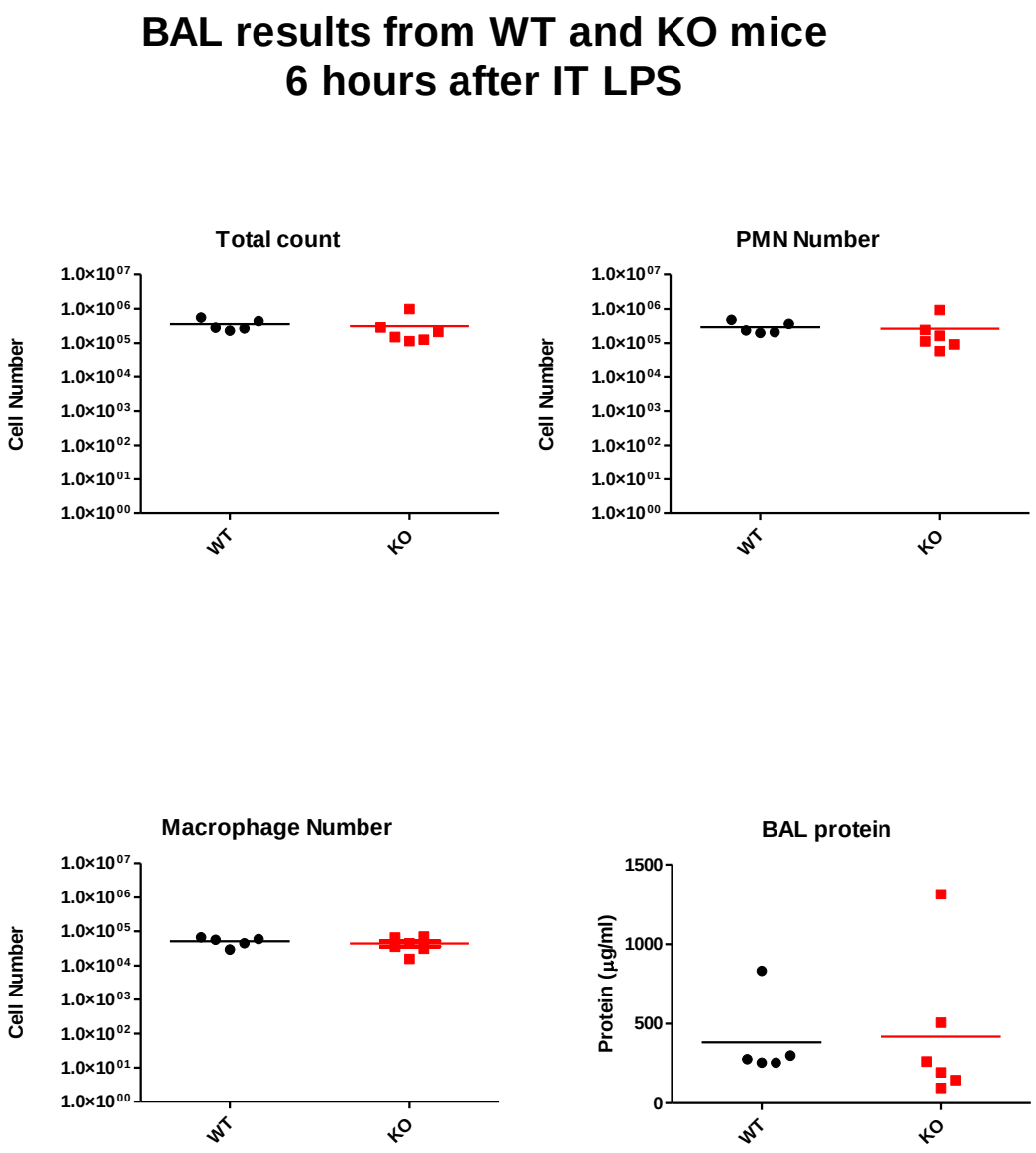


Figure 5-10 Bronchoalveolar lavage results from WT and KO animals given 50 µg intratracheal LPS and sacrificed at 6 hours post-instillation. Each data point represents an individual animal. Bar equals mean value. Groups compared by Mann-Whitney test. No significant differences seen.

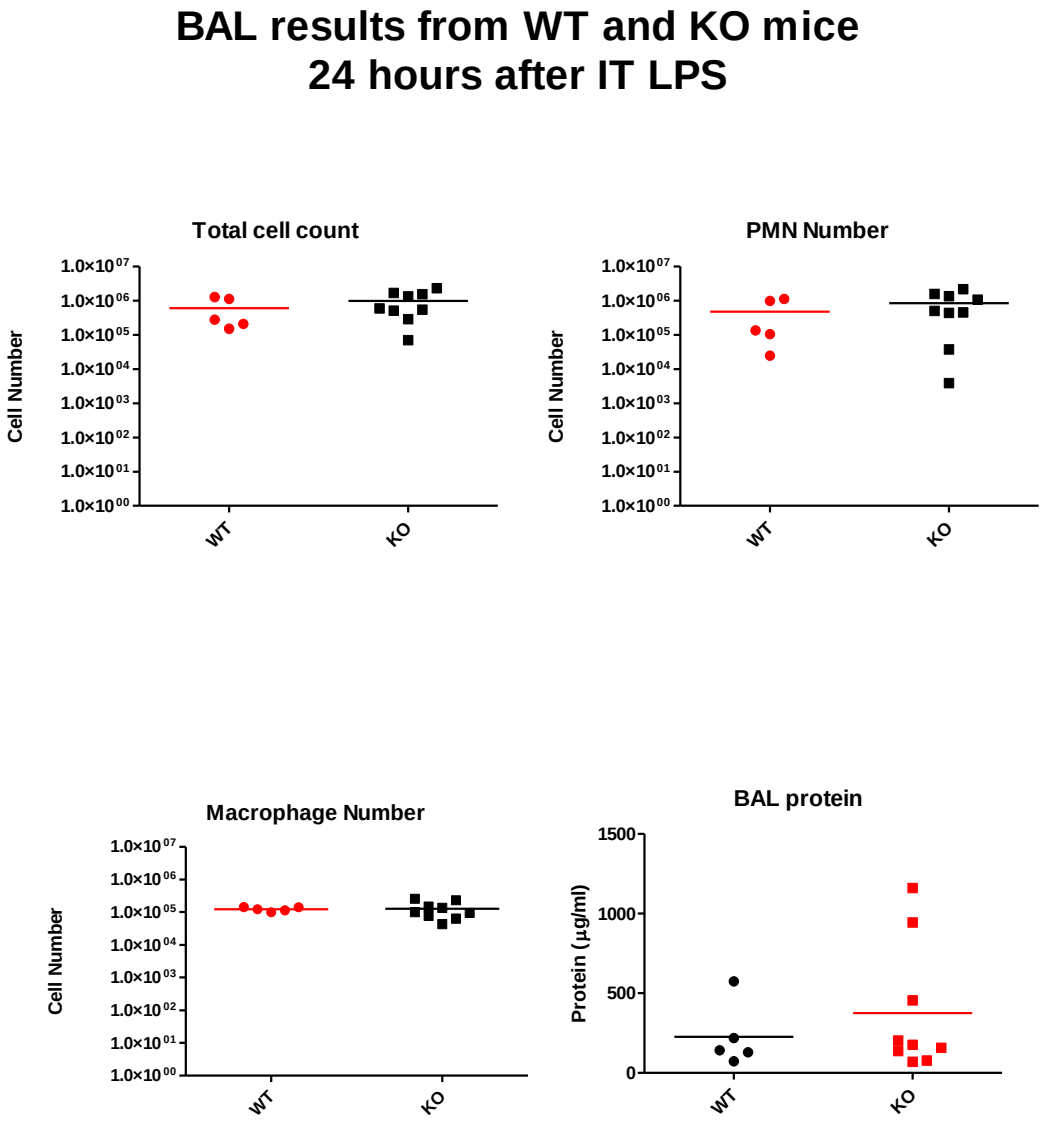


Figure 5-11 Bronchoalveolar lavage results from WT and KO animals given 50 µg intratracheal LPS and sacrificed at 24 hours post-instillation. Each data point represents an individual animal. Bar equals mean value. Groups compared by Mann-Whitney test. No significant differences seen.

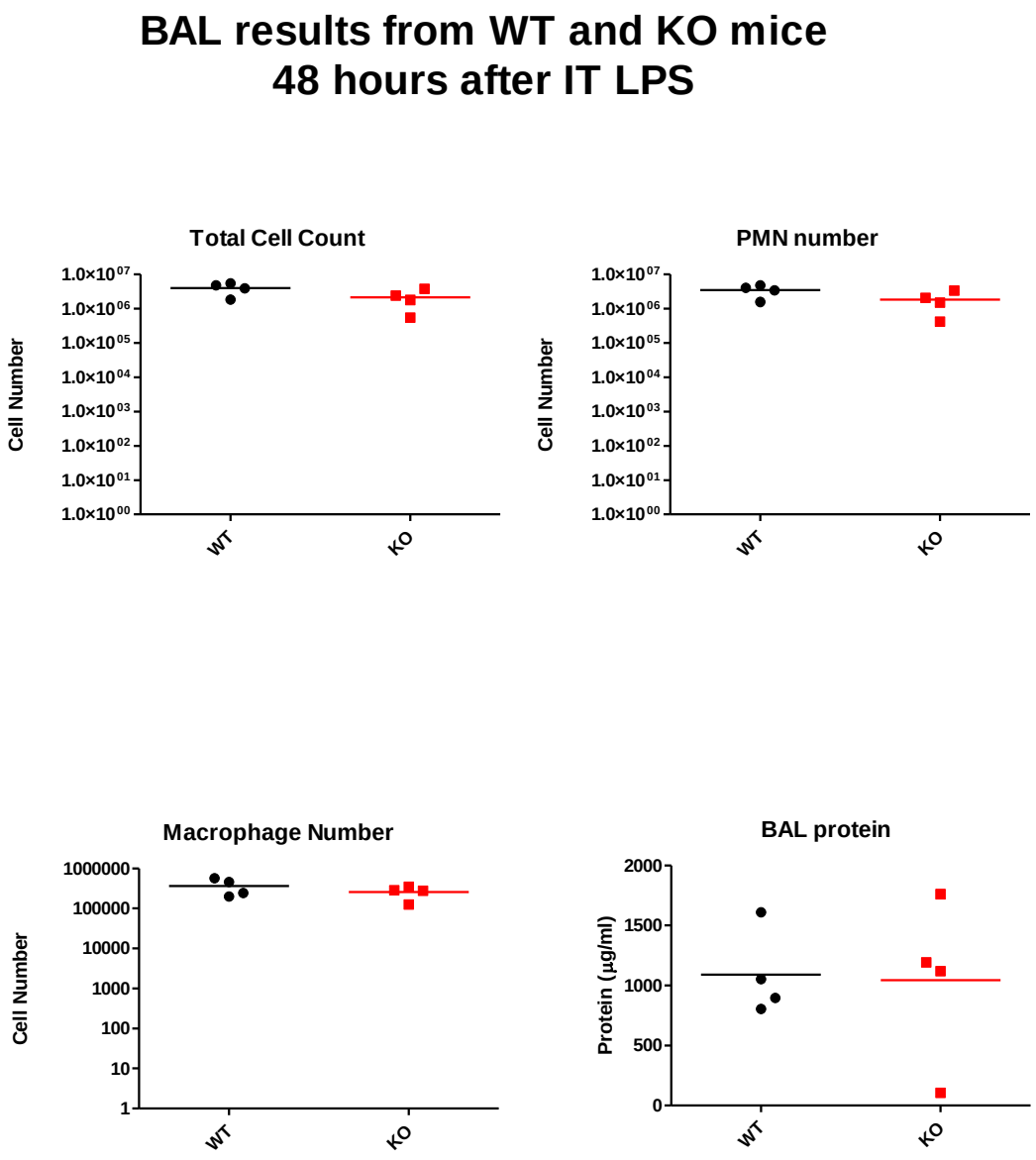


Figure 5-12 Bronchoalveolar lavage results from WT and KO animals given 50 µg intratracheal LPS and sacrificed at 48 hours post-instillation. Each data point represents an individual animal. Bar equals mean value. Groups compared by Mann-Whitney test. No significant differences seen.

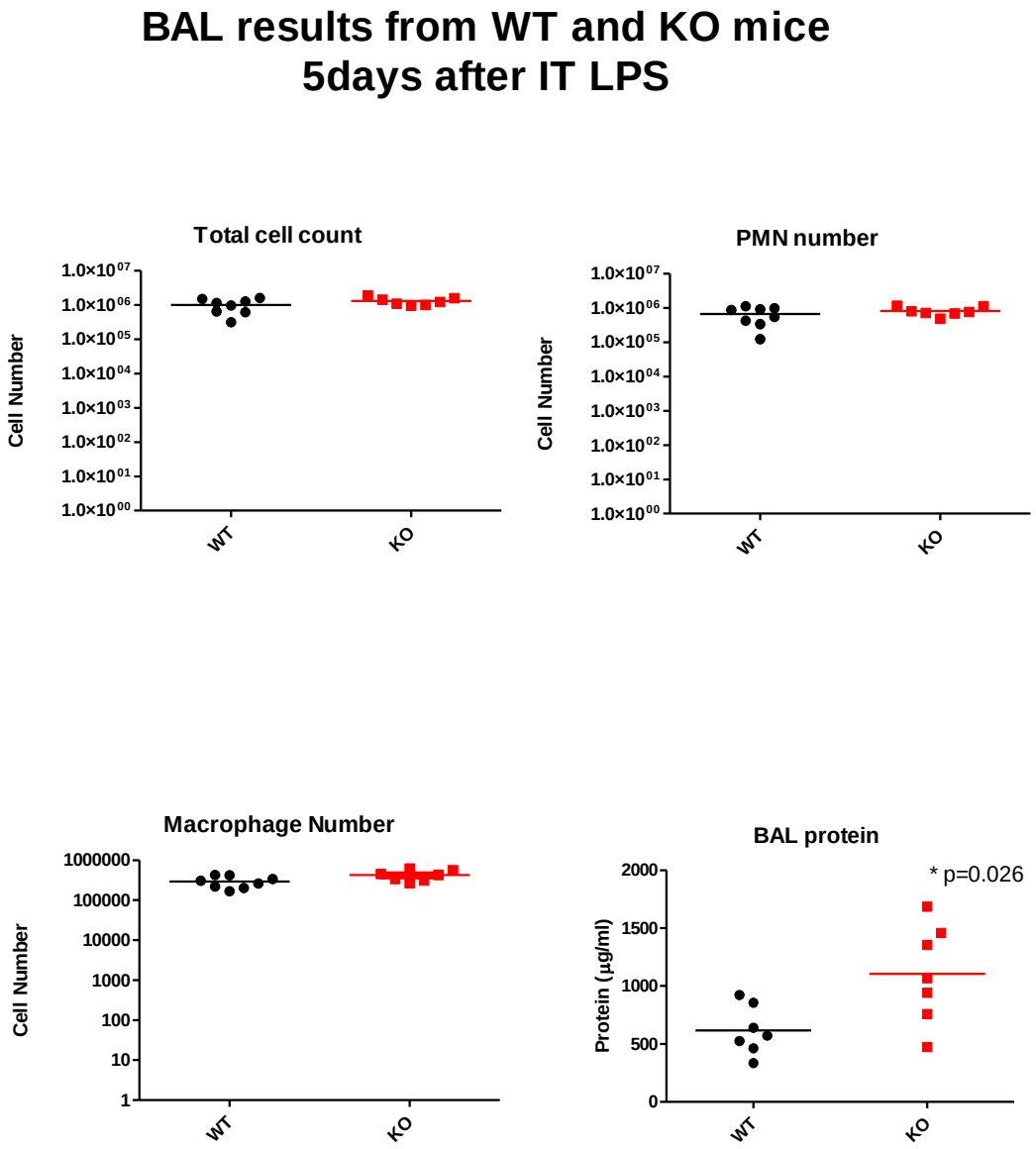


Figure 5-13 Bronchoalveolar lavage results from WT and KO animals given 50 µg intratracheal LPS and sacrificed at 120 hours (5 days) post-instillation. Each data point represents an individual animal. Bar equals mean value. Groups compared by Mann-Whitney test.

The overall similarity in the severity of the injury sustained by WT and KO mice is reflected in the change in percent body weight over the 5 day course of one experiment (Fig5-13).

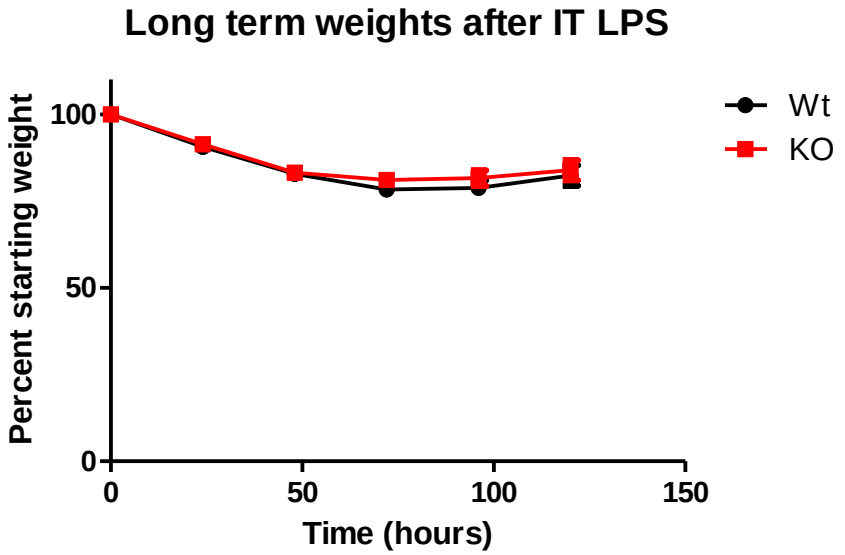


Figure 5-14 Change in body weight over 5 days following instillation of 50 µg intratracheal LPS. Error bars denote standard error.

This shows that the nadir of bodyweight is reached by day 3 and there is a subsequent increase in weight as the inflammatory response subsides corresponding with the BAL results.

5.2.8 BAL release of TNFα is not impaired in Hif-1α myeloid deficient animals

TNFα is known to be a potent neutrophil chemoattractant and its elevation in BAL fluid is thought to be important in the localisation of neutrophils to the alveolar space in lung injury. The release of TNFα in response to intratracheal LPS was therefore studied (5-14). There was no significant difference between WT and KO animals although the levels at 2 hour level in WT animals was 50% higher than in KO animals.

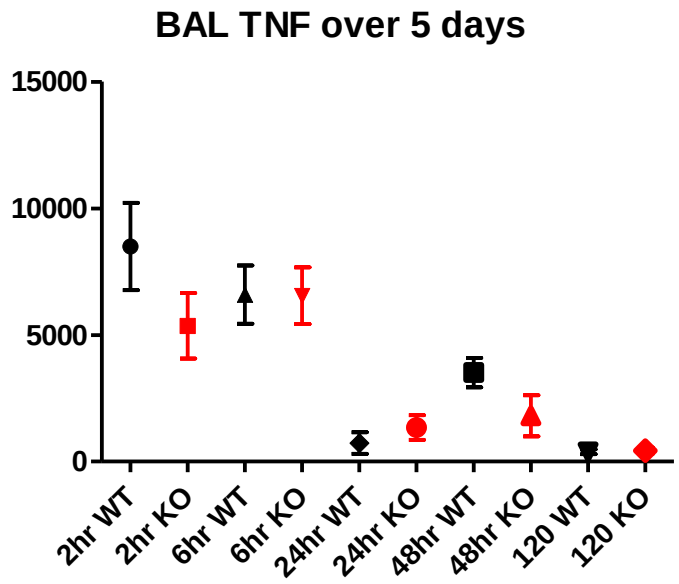


Figure 5-15 Bronchoalveolar lavage TNF from WT and KO animals given 50 µg intratracheal LPS for the time points shown. Each point is mean value. Error bars denote standard error.n=4-9 animals per point.

5.2.9 Hif-1α myeloid deficient animals show reduced pulmonary nitrosylation in response to LPS.

Quantitatively the response to intratracheal LPS is similar in WT and KO mice. However it is known that the bactericidal effectiveness of KO myeloid cells is reduced even though the inflammatory influx seen may be similar [218]. Since the generation of NO is thought to have an important role in the bactericidal ability of myeloid cells and in the development of ALI the degree of protein nitrosylation (a marker of NO mediated tissue damage) was assessed by immunohistochemistry [236]. IHC showed that there was greater staining of pulmonary tissue in WT than KO animals consistent with a greater generation of reactive nitrogen species in WT than KO animals.

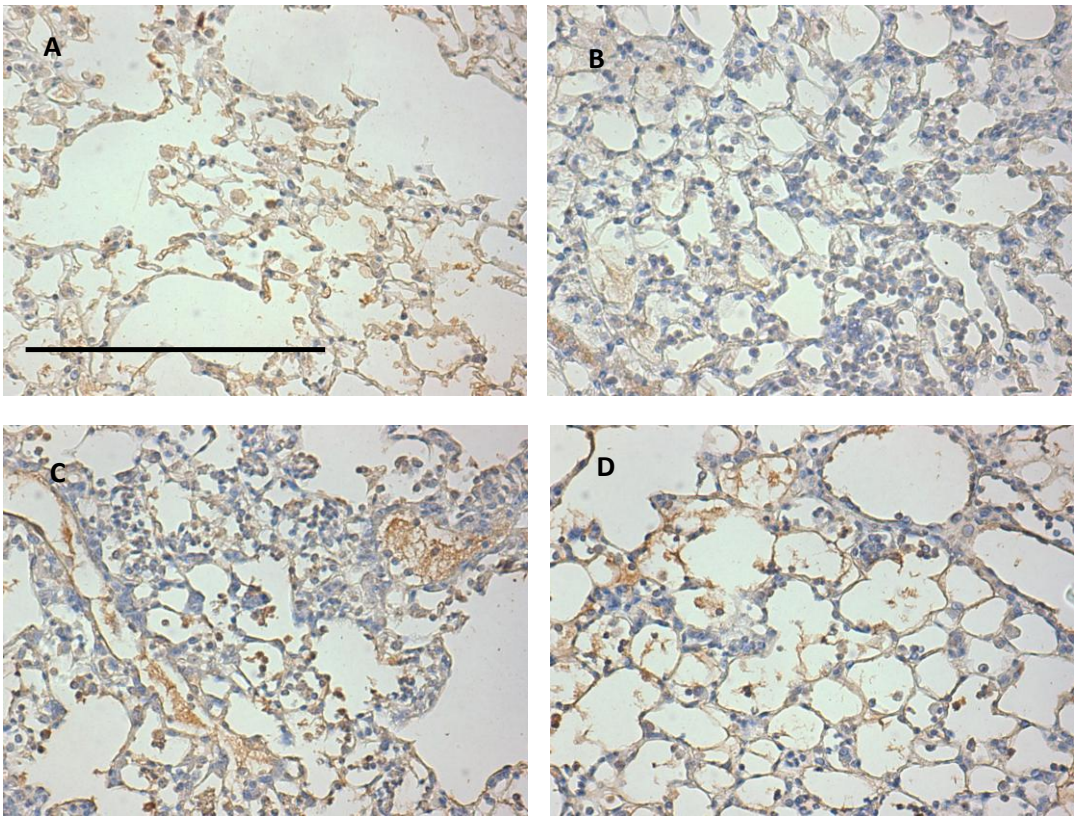


Figure 5-16 Nitrotyrosine Immunohistochemistry of lungs from Hif WT and KO animals showing reduced protein nitrosylation at both 24 hours and 5 days in KO animals after LPS instillation. A- WT 24hours post LPS. B-KO 24 hours post LPS. C-WT 5days post LPS. D-KO 5 days post LPS. Magnification x200. Graticule=100µm.

It was not possible to measure reactive nitrogen species directly in BAL fluid due to the presence of haemoglobin in some lavages [237] resulting from alveolar haemorrhage [192]. However, the presence of nitrosylated proteins is accepted as an indicator of reactive nitrogen generation in studies of ALI [238].

5.2.10 Maximal Pulmonary Hypoxia is seen at 5 days

Pimonidazole is a compound which binds to hypoxic proteins irreversibly and can therefore be used as a marker of tissue hypoxia by detecting it using immunohistochemistry using a commercially available kit. Using this method the development of tissue hypoxia was studied in lungs instilled with LPS in animals exposed to LPS for 24 or 120 hours. This experiment showed that there was minimal tissue hypoxia at 24 hours post-instillation but that by 5 days there were multiple hypoxic cells which were predominantly inflammatory leucocytes (figure5-15).

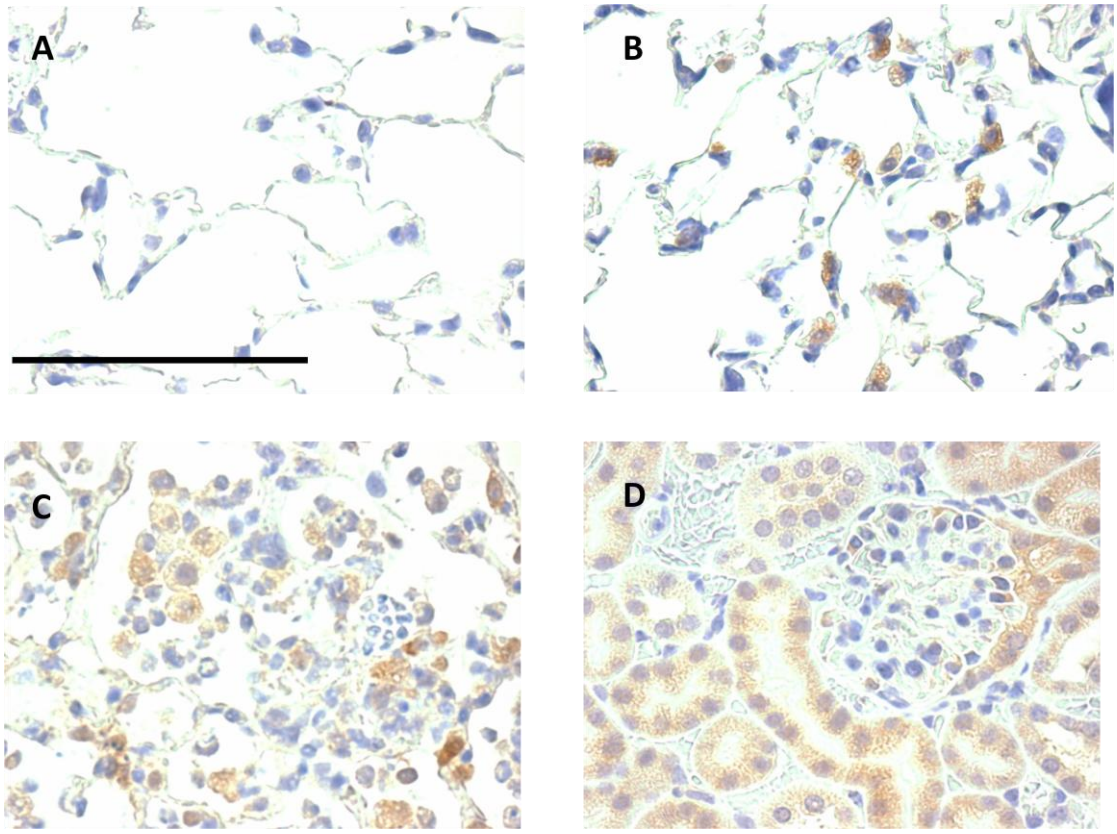


Figure 5-17 Immunohistochemical straining for Pimonidazole in lungs from Control (A), 24 hours IT LPS (B) or 120hours IT LPS (C)showing increased staining centred on inflammatory cells at day 5. Section D is kidney confirming the ability of Pimonidazole to discriminate normoxic tissue (Glomeruli) from hypoxic (renal tubules). Magnification x200 Graticule=100µm.

5.2.11 Hif-1α depleted Bone Marrow Derived Macrophages produce less Nitric oxide in response to Endotoxin

Bone Marrow Derived Macrophages (BMDM) were cultured by established techniques and then exposed to LPS. Neither unstimulated WT nor KO BMDM generated significant quantities of nitrite over the study period. In response to LPS both WT and KO BMDM generated increased amounts of nitrite but KO BMDM generated about half the amount WT cells were able to.

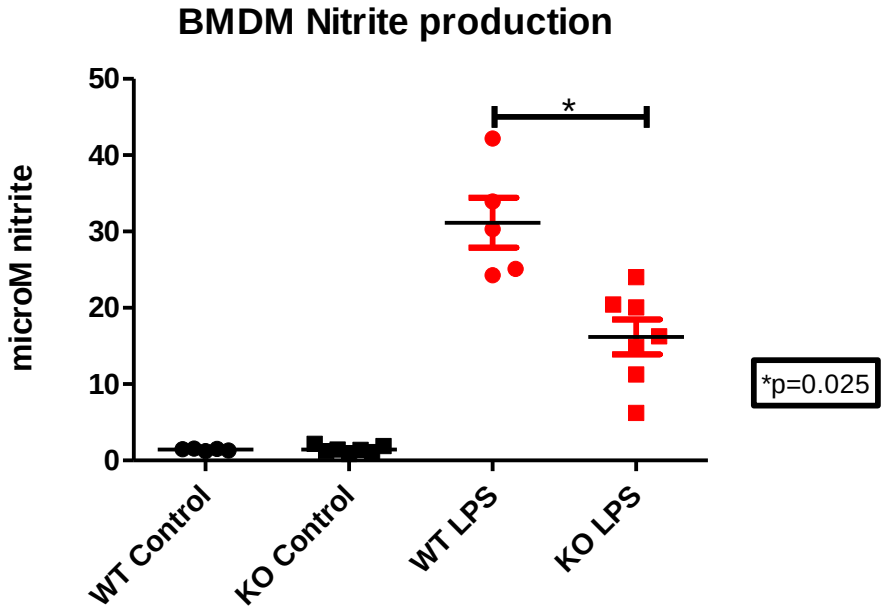
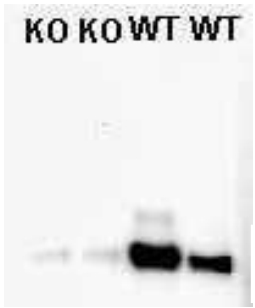


Figure 5-18 Nitrite release from Bone Marrow Derived Macrophages from WT or KO animals exposed to Saline or 1µg LPS for 24 hours. Each point represents mean result of triplicate wells from one animal. Bar at mean value. Statistical difference shown by MannWhitney test.

To confirm these results a Western blot was performed to study the generation of iNOS protein in response to LPS. It can be seen that the amount of iNOS present in LPS stimulated Hif-1α KO BMDM is greatly reduced compared to WT.



iNOS band present in WT but not KO animals after LPS stimulation

Figure 5-19 iNOS protein from KO or WT animals BMDM after LPS stimulation.

This confirms the key role of myeloid Hif-1α in the generation of reactive nitrogen species in response to bacterial infection.

The response to bacterial infection is controlled by the release of cytokines from myeloid cell, one of which TNFα, is thought to have a central role in the generation of the immune response. To determine whether this regulatory function of myeloid cells is altered in Hif-deficient cells BMDM from WT and KO animals were exposed to LPS for varying periods of time and the amount of TNFα released into the supernatant was measured.

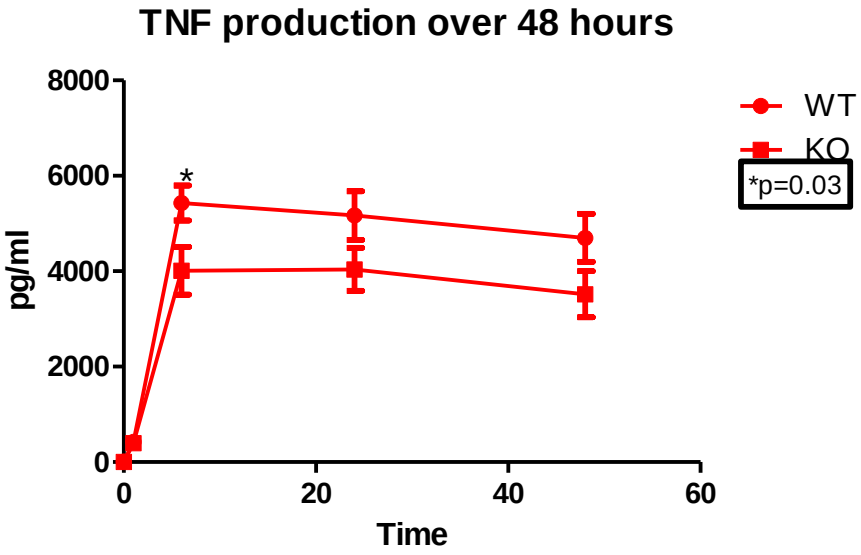


Figure 5-20 Release of TNF in response to 1µg LPS by WT or KO BMDM. Mean result of 3-7 animals per time point shown. Error bars denote standard error. P values calculated by Mann Whitney test.

At 6 hours WT BMDM had released significantly more TNFα than WT. However there was no difference at 1 hour and this effect was lost at 24 and 48 hours.

5.2.12 VHL mice show no difference in response to LPS after 5 days.

Some mice, which over express myeloid Hif-1α (known as VHL) due to a conditional depletion of the von Hippel Lindau protein, became available towards the end of the project. An experiment was performed in which VHL mice and their WT littermates were exposed to intratracheal LPS and then underwent BAL at 5 days (figures 5-18 and 5-19)

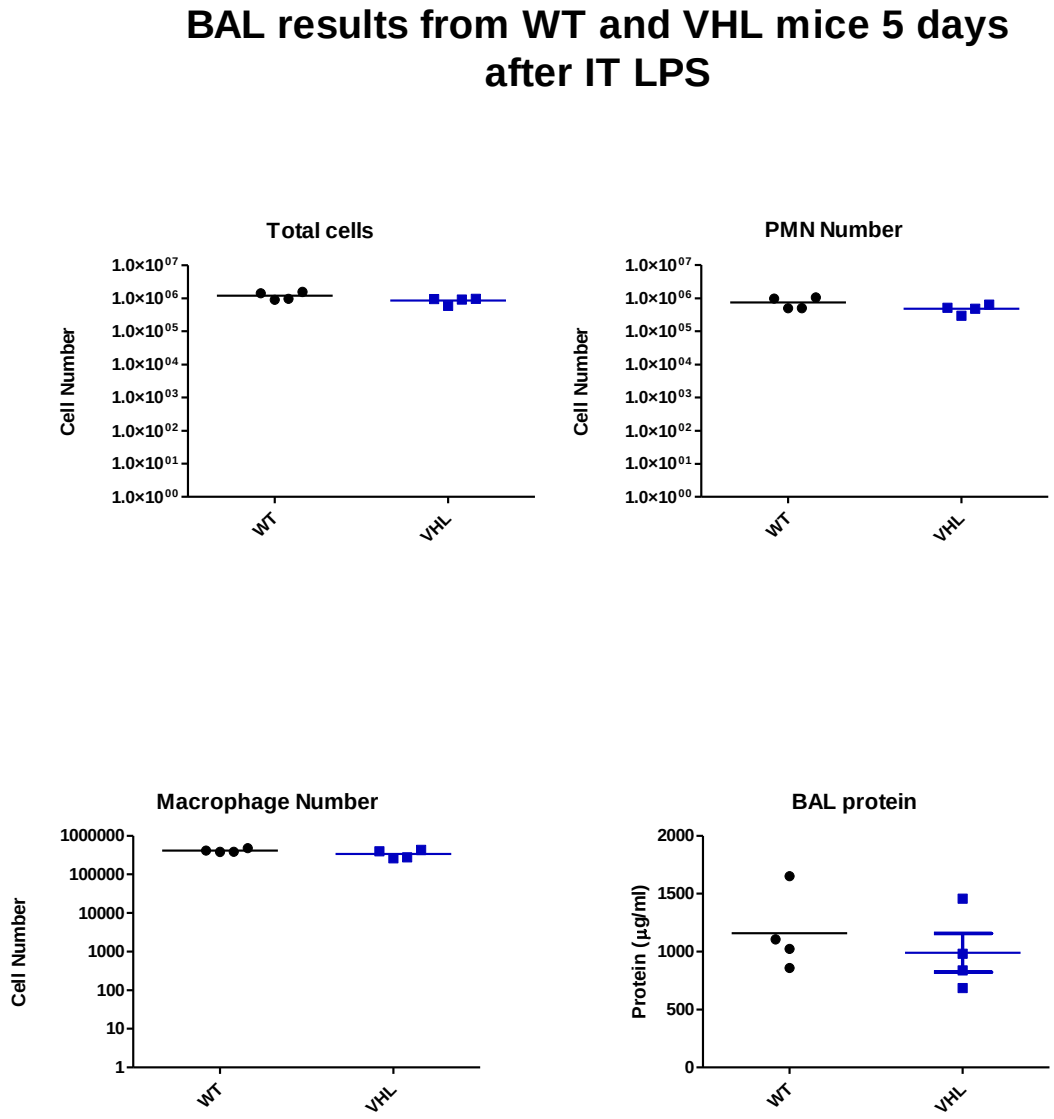


Figure 5-21 Bronchoalveolar lavage results from WT and Hif-1 over-expressing (VHL) animals exposed to 50µg intratracheal LPS for 120 hours. Each point represents an indivaidual animal, bar is mean result. No difference between wild type and knockout animals examined by Mann Whitney test.

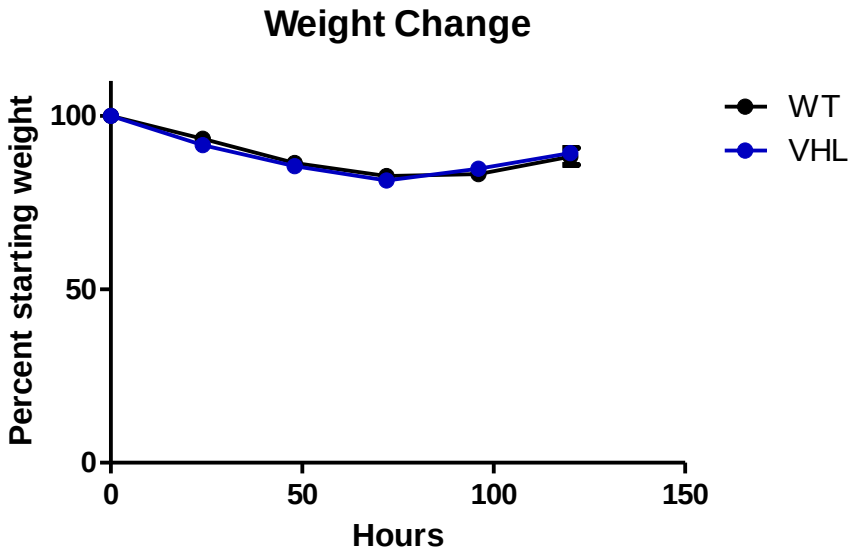


Figure 5-22 Percent weight change in WT and VHL animals exposed to 50µg intratracheal LPS over 5 days. Mean values shown. Error bars denote standard error.

It can be seen that over the 5 day period both groups of animals had a similar change in body weight and that BAL performed on day 5 gave similar results. This supports the observations in the Hif-1α deplete mice that they have a similar systemic response to i.t. LPS as wild type animals.

5.3 Discussion

This chapter has been concerned with the characterisation of the Hif-1α myeloid cell conditional knockout mouse gifted from Prof. R Johnson and exploring its response to intratracheal LPS as a model of Acute Lung Injury.

The presence of the floxed alleles in the genomic DNA of the animals was confirmed and the efficiency of the process was shown to be high in terminally differentiated alveolar macrophages. The deletion efficiency is slightly higher in our alveolar macrophage population than was reported by Cramer et al [89] in a population of peritoneal macrophages. Macrophages are an heterogeneous population [239;240] of cells. By immunohistochemistry we have shown that alveolar macrophages in these animals continue to express the Cyclization recombination (Cre) gene in their nuclei. Persistent expression of this enzyme allows for the deletion of the floxed segment of target gene occurring at any stage of the cell's lifecycle once Lysozyme M is expressed. The expression of Lysozyme M is progressively turned on during myeloid cell development [241]. Therefore it could be hypothesised that more mature cells have a greater likelihood of having the floxed segment deleted as they have expressed Cre for longer and therefore there is a greater likelihood that the Cre enzyme has acted on the floxed segment. Overall the deletion of the Hif-1α target sequence is highly efficient in this animal. The use of a cell specific promoter is a relatively new approach to studying the effect of a specific cell population. Previous work has used liposomal clodronate [242] to deplete the alveolar macrophage population. However, this approach does not result in a 100% depletion of the original population, does not affect parenchymal cell and most importantly in this field generates a pulmonary

inflammatory response [76;243-245]. Therefore the hope of using cell specific transgenic animals is that these confounders can be avoided.

Following the confirmation of the genetic mutation in the animals it has been shown that the wild type and knock out littermates have similar bronchoalveolar cell populations following saline instillation. This confirms the findings of Cramer et al [89] and Peysonneaux et al [218] that the composition of the resting tissue populations of inflammatory cells are not affected in this strain of mouse. It is appreciated that the activity of Hif-1α in these animals is reduced in all cells of the myeloid lineage [89;227]. However, there is no absolutely specific macrophage promoter available that would allow this problem to be circumvented. It is possible, having depleted the mouse's own neutrophil population (e.g. by using anti-GR1 antibodies), to transfuse mice with neutrophils harvested from the bone marrow [246-248] of donor animals. Similarly it is possible to transfer macrophages from one animal to another. It would therefore be possible, if a significant effect on injury or repair was seen in the Hif-1α knockout animals, to try and tease out the importance of each myeloid cell type by the adoptive transfer of either wild-type neutrophils [248] or macrophages[249] to knockout animals. However in the data presented in this work the effect of Hif-1α deletion appears to be minimal in the development and progression of ALI.

Subsequently in an experiment only using KO animals it was shown that KO animals are able to generate an inflammatory response in response to intratracheal LPS that is not seen following intratracheal saline when compared to unchallenged mice.

The main aim of this chapter was to study the response of Hif-1α KO mice and their WT littermates to intratracheal LPS. Over a range of time points we have shown that both WT and KO animals develop an intense neutrophilic alveolitis in response to i.t.

LPS. The response peaks around 48 hours after challenge and is beginning to resolve by day 5 with a reduction in PMN number and increase in animal body weight.

The results of BAL analysis from WT and KO animals are similar at most timepoints with 2 notable exceptions. At 2 hours after challenge there are fewer PMN in the BAL of KO animals than WT (KO 7485 ± 925.7 N=4 v WT 12410 ± 1231 N=4 p=0.0186). This difference is lost by 6 hours however, and the severity of neutrophil inflammation is similar at all subsequent timepoints studied. Cramer et al reported that these mice showed deficient inflammatory responses to both topically applied SDS and in a model of passively induced arthritis. However, similar to our findings Peyssonnaux et al [218] from the same group found that the severity of the inflammatory cell response to a skin infection model did not differ between WT and KO animals. They hypothesised that the stimulus to neutrophil migration induced by bacterial infection was such that it overcame the contribution to migration that Hif-1α appeared to play in their earlier study. Hif-1α is central to the generation of ATP in myeloid cells and our results would suggest that the energy requiring migration of neutrophils is impaired in these animals resulting in slower but still effective localisation to inflammatory stimuli. In order to further investigate this finding it would be of value to study the inflammatory response at an even earlier timepoint (30mins to 1 hour) to see if the difference persists. In addition the ability of KO BM derived PMN to migrate in response to classical chemotactic stimuli should be assessed using modified Boyden chambers.

The other difference seen is a 5 days where by this stage the BAL protein level of WT animals is staring to fall while in KO animals it remains elevated (WT 615.6 ± 79.51 N=7 v KO 1106 ± 159.9 N=7, p=0.0178). The reason for this difference is unclear but

suggests that Hif-1α may play a role in limiting responses to inflammatory insults, turning off injury at the time when pulmonary hypoxia as assessed by Pimonidazole binding is greatest in this series of experiments. The identification of areas of hypoxic pulmonary tissue is important here as it is not widely appreciated that the abnormal, injured lung can have areas of hypoxia as a result of impaired ventilation. It is recognised in thoracic surgery that pulmonary ischaemia occurring as a result of loss of ventilation and perfusion of the collapsed lung [51;53;54] is an important stimulus to injury. However, areas of regional collapse and consolidation seen in the critically ill [8] are also likely to result in hypoxic tissue, even though the fraction of inspired oxygen may approach 1.0.

Hif-1α has been shown to have anti-inflammatory effects in T-lymphocytes [250] while in animal models of colitis increasing Hif-1α levels pharmacologically has been shown to be beneficial [217;219] although the mechanisms are unclear. Furthermore it has been shown that oxygen inhibits tissue protection in ALI and exacerbates tissue injury [251] while hypoxia for 48 hours was protective (if the animal survived the initial hypoxic exposure). This was proposed to be due to the effects of oxygenation on adenosine mediated tissue protection. Adenosine generated in the protective responses requires the glycolytic generation of ATP the key enzymes for which are Hif-1α regulated. Therefore Hif-1 may have a role in the resolution of lung injury that was not expected from initial studies. An important off switch of inflammation, the release of adenosine acting on the A_{2A} Receptor, modulated by Hif-1α regulated production of up-stream mediators, may be impaired resulting in prolonged inflammatory damage.

Following the resolution of injury over a more prolonged period, to complete restoration of normal pulmonary cell populations would support this hypothesis. Furthermore this observation raises the possibility of a protective effect from Hif-1 expression in other, more chronic forms of lung injury, such as interstitial fibrosis, which could be tested using the well established bleomycin model of murine lung fibrosis. The use of specific $A_{2A}R$ agonists such as *CGS 21680* which are commercially available to restore the reparative process in KO animals would confirm the role of Hif-1 mediated adenosine generation in these animals.

Reactive nitrogen species have been suggested to play a role in the generation of ALI. Nitric oxide Synthase II (inducible nitric oxide [iNOS]) is Hif-1α regulated. Using immunohistochemistry we have shown that the generation of nitrosylated proteins in the lungs of the KO mice is reduced. Further using harvested BMDM we have shown that the lack of generation of nitric oxide in these animals is due to the expected lack of induction of iNOS [218]. In agreement with Peyssonnaux et al [218] we also found that the generation of TNF was reduced in KO BMDM although this difference was only seen at 6 hours after LPS exposure. Again, as in chapter 4, it is not known if there is any difference between solTNFα and mTNFα expression in this system. The difference in TNFα in medium at 6 hours could be the result of reduced activity of TACE. It could therefore be hypothesised that although generation of TNFα might not be altered by Hif-1α deficiency the reduced medium release may be explained by reduced cleavage of the 26kDa form [222]. However, it should be noted that there would not appear to be a biological effect of this potential system in the model of ALI as cell infiltration and BAL protein at 6, 24 and 48 hours are unaffected.

The late availability of mice over-expressing Hif (VHL mice) meant that only a single experiment could be performed using them. The over-expression of Hif did not generate a more severe inflammatory response in these animals measured by change in body weight. BAL at 5 days showed similar inflammatory cell profiles between wild type and Hif over-expressing animals.

5.4 Future work

This chapter has shown that the acute inflammatory response to intratracheal LPS is not significantly dependent on myeloid cell Hif-1α. Presumably other mediators such as NF-κB and AP-1 are more central to the response. However the data has raised the possibility that Hif-1α has a role in the down regulation of the generated response and studying the later stages of the repair process would be valuable. Further if Hif-1α has a role in the inhibition of inflammatory responses then assessing the role of myeloid cell Hif-1α in more prolonged inflammatory models such as Lung fibrosis would seem appropriate.

Chapter 6

Manipulation of Endotoxin induced lung injury by a Prolyl-hydroxylase inhibitor

6.1 Introduction

Results discussed in the preceding chapter suggest that in the generation of acute lung injury manipulation of myeloid cell Hif-1α seems to have minimal effect on the quantity of the response although the quality of the response is changed.

Studies have shown that the pharmacological manipulation of Hif-1α in isolated cells [252]or in whole animals [217;219;253] can alter the response to insults. Furthermore the manipulation of downstream components of Hif-1 controlled pathways can alter the response to inflammatory insults [221;254].

Dimethyloxalylglycine (DMOG) is an inhibitor of prolyl hydroxylases, key enzymes in the control of Hif-1α metabolism [120]. DMOG and other PHD inhibitors given systemically can act as a preconditioner to hypoxic challenge, increasing the tolerance of animals to hypoxia [253]. Recently in models of detergent induced colitis two separate groups of investigators have shown that the systemic administration of PHD inhibitors can reduce the severity of the inflammatory injury [217;219]. Both groups used a dosing schedule comprising several treatments with a PHD inhibitor before and after administration of the insult. Based on our experiences with DMOG in vitro and our interest in the possible manipulation of the response to pulmonary inflammatory insults we undertook a study of the effect of local PHD inhibition on the pulmonary response to endotoxin.

Because of the unexpected nature of the insult in ALI it was decided that pre-treatment of the animals would not give clinically useful information. It was therefore decided to administer the DMOG simultaneously with the noxious agent, endotoxin. Furthermore it was decided to administer the DMOG intratracheally in order to allow

us to study the pulmonary response to the agent and minimise its systemic effects. This provides a clinically useful scenario where the administration of agents (e.g. Nitric oxide) via the endotracheal tube to patients with ALI has been shown to modulate the inflammatory response. Furthermore simultaneous administration required the animal to receive only one dose of anaesthetic to allow i.t. dosing. A dose of 8mg of DMOG was chosen as this reflects the dose used by other groups in the literature [217]. For these experiments the DMOG was dissolved in Phosphate buffered saline to avoid any effect from vehicles.

The hypothesis under investigation here was that manipulation of Hif-1 by the use of intratracheal DMOG would alter the response to intratracheal endotoxin.

The aim of this chapter was to use the previously described model of pulmonary inflammation generated by intratracheal endotoxin to study the effect of simultaneous administration of DMOG.

6.2 Results

6.2.1 Simultaneous DMOG and LPS does not reduce neutrophil infiltration in ALI.

C57/Bl6 mice were anaesthetised and either exposed to i.t. LPS or LPS and DMOG. Animals were allowed to recover for 24 hours before sacrifice and BAL.

Both groups of animals lost approximately 15% of their body weight following dosing confirming the severe nature of the inflammatory insult (Figure 6-1)

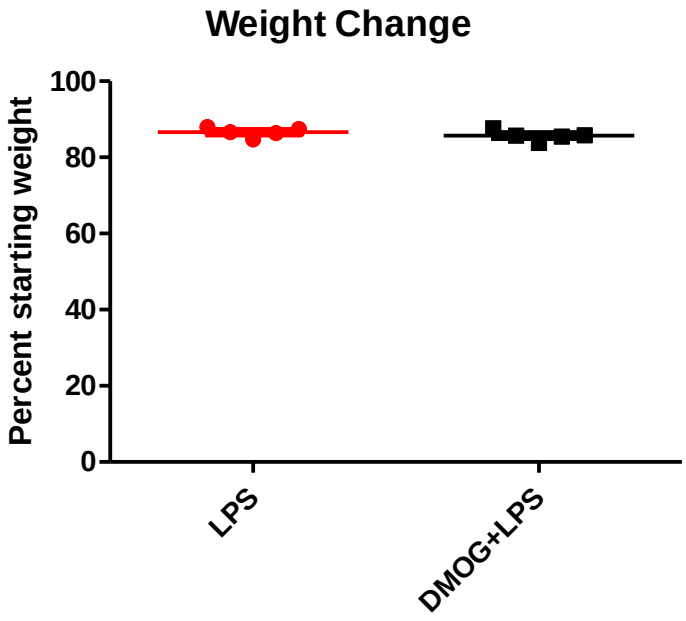


Figure 6-1 Change in body weight in animals exposed to intratracheal LPS or concurrent LPS and DMOG.

The results of BAL analysis from the animals are shown in Figure 6-2

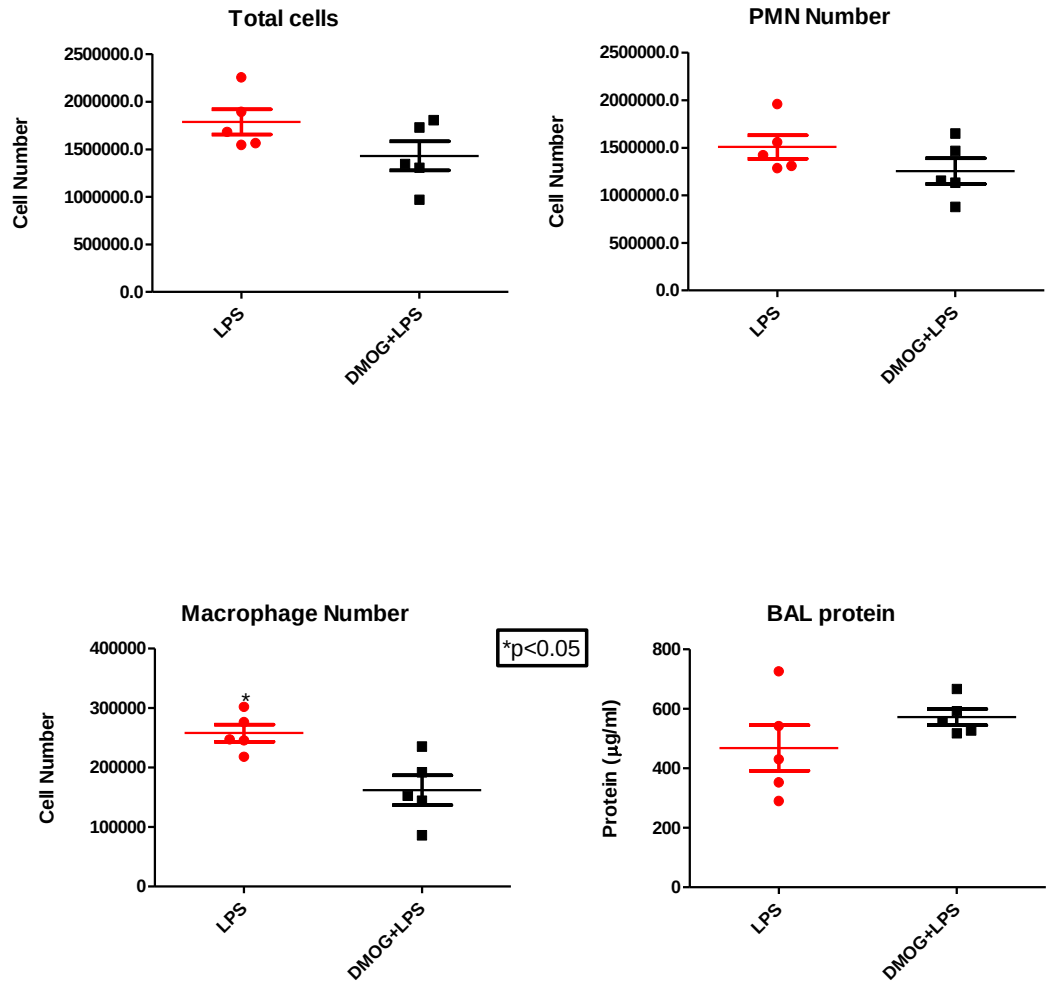


Figure 6-2 Bronchoalveolar lavage results from animals exposed to intratracheal LPS or concurrent LPS and DMOG. Error bars denote standard error.

As expected the administration of i.t. LPS resulted in an intense neutrophilic alveolitis. The simultaneous administration of DMOG at a dose known to systemically inhibit PHDs and therefore increase Hif levels did not alter the neutrophil influx nor the damage to the alveolar capillary integrity, evidenced by the similarity in BAL protein levels. The number of macrophages in the BAL of animals given DMOG + LPS was significantly reduced compared to that in LPS treated animals alone but the significance of this result is unclear.

6.2.2 Local administration of DMOG increases iNOS expression in BAL cells.

In the previous chapter it was shown that the quality of the inflammatory response was different in animals with reduced myeloid Hif-1α expression, specifically the reduction in iNOS expression.

It was decided to study the generation of iNOS by the alveolar infiltrate. To do this the cells obtained from BAL were counted and the remaining cells were then lysed and their cellular protein was collected. Aliquots of cytoplasmic protein were then analysed by Western blot (Figure6-3)

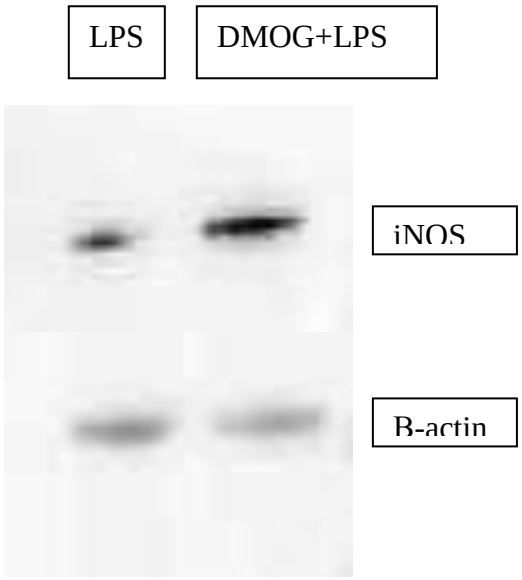


Figure 6-3 Western blot showing expression of iNOS protein in alveolar cells from animals treated with intratracheal LPS or concurrent LPS+DMOG

It can be seen that in the cytoplasmic extract from the dual treated cells there is an increase in the expression of iNOS protein. β-actin confirmed similar loading of the gel.

Unfortunately as previous discussed it was not possible to measure BAL nitrite levels but these would be expected to be higher in the DMOG+LPS samples than in the LPS alone.

6.2.3 Increased Nitrotyrosine staining in DMOG+LPS treated animals.

As a surrogate for reactive nitrogen species levels the pattern of protein nitrosylation in the lungs of animals treated with LPS alone or LPS+DMOG was studied by immunohistochemistry.

As expected from the result of the iNOS Western blot lungs from animals treated with both LPS and DMOG showed increased deposition of nitrosylated protein compared to LPS alone (Fig 6-4).

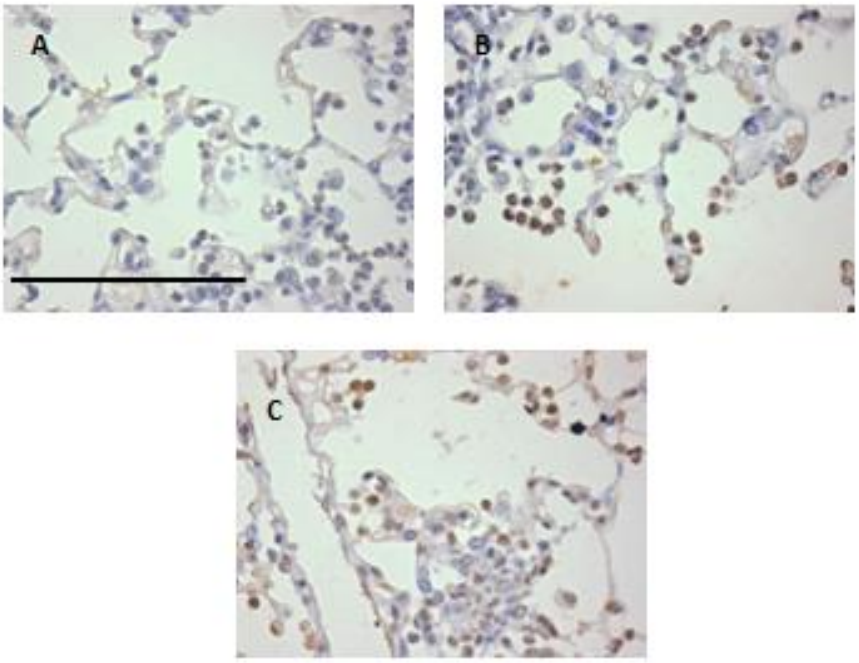


Figure 6-4 Nitrotyrosine immunohistochemical stain in animals exposed to intratracheal LPS (A) or LPS+DMOG (B and C). x200. Graticule=100µm.

LPS treated animals showed deposition of nitrosylated proteins on some alveolar walls and also inflammatory cells. In the animals treated with DMOG+LPS there was more intense staining on inflammatory cells.

These results again suggest, in a similar pattern to the myeloid deplete animals that although the quantity of inflammatory infiltrate is not affected by manipulating Hif levels the quality of the response is.

6.3 Discussion

The recent availability of compounds which can pharmacologically manipulate the Hif-1 pathway raises the potential for their clinical use in modulating inflammatory responses. Dimethyloxalylglycine is a prolyl hydroxylase inhibitor which has been shown to have beneficial effects when given to a murine model of Inflammatory Bowel Disease [217]. Similar results were found by an independent group using a related compound [219] in another model of murine colitis.

In work previously described here, DMOG was shown to increase the generation of Nitric oxide in response to LPS while the release of TNFα was reduced from murine alveolar macrophages. Furthermore using the Hif-1α myeloid deplete animals it was shown that the nature of the inflammatory response to intratracheal LPS was altered in these animals. It was therefore decided to investigate the effect of DMOG administration on the pulmonary inflammatory response to LPS. In order to generate clinically useful information it was decided to administer a single dose of DMOG at the same time as the insult representing as near as possible the original clinical scenario this project was interested in of a critically ill patients and the early resuscitation/treatment phase. Furthermore the DMOG was administered intratracheally to minimise any systemic effects. The DMOG given here will therefore have actions on the resident alveolar epithelium, alveolar macrophages and recruited inflammatory cells.

The single dose of DMOG had no effect on the magnitude of the neutrophilic infiltrate generated by the LPS. Similarly there was no difference in the degree of BAL protein between LPS and LPS+DMOG. This is a significant clinical finding as the DMOG could be expected to increase epithelial Vascular Endothelial Growth

Factor, a Hif mediated transcription factor which may increase alveolar permeability (although prolonged dosing did not lead to an increase in colonic permeability in the models of colitis [219]). As predicted by in vivo data the DMOG increased the levels of iNOS protein in inflammatory cells measured by Western blotting and there was an increase in the generation of nitrosylated proteins seen by immunohistochemistry.

The data here raises the possibility that the administration of DMOG near to an infective insult may be beneficial as there would appear to be little effect on the recruitment of inflammatory cells, there is no gross deleterious effect on the alveolar epithelial-capillary integrity but there is an increase in the ability of the inflammatory cells to generate a bactericidal response measured by reactive nitrogen species.

There are two future areas of research based on the data. First it would be valuable to study the effect of delayed administration of DMOG to the ALI response. Cummins et al [217] in their model of colitis found that DMOG increased the repair following insult removal and the possibility of a therapeutic option for ALI is an exciting one. Secondly the possible beneficial of DMOG in an infective pulmonary insult deserves investigation. The results above raise the possibility that administration of DMOG to a model of pneumonia may result in an increase in bacterial killing as a result of increased Hif activation [218] without an increase in pulmonary damage.

Chapter 7

General Discussion and Conclusion

7 Discussion and Conclusion

Acute Respiratory Distress syndrome remains a significant cause of morbidity and mortality in critically ill patients. Despite much investigation there has been no specific therapy shown to improve outcome and treatment remains supportive with the aim of minimising further lung damage by a protective ventilatory strategy. ARDS therefore remains a frustration to investigators, as despite a number of possible approaches to treating the disease, it seems that the complexity and overlap seen in the system prevents the identification of a single therapeutic intervention that dramatically alters the course of the illness. Indeed by the time the syndrome is clinically recognisable the over-exuberant immune response is underway and it would seem obvious that a single agent is unlikely to be able to target all the processes occurring.

This therefore raises the possibility of trying to identify those at risk of ALI and treating them in order to attenuate any lung injury likely to develop. Those at risk of ALI are well recognised [12] and work in this institute has shown that early mediators can be identified that predict the development of ARDS [29]. Furthermore it would seem that these mediators are linked to early indicators of hypoxia [58] thus raising the possibility that hypoxic signalling contributes to the development of the organ injury.

This thesis therefore aimed to study the role of a specific hypoxic signalling pathway, that of hypoxia inducible factor-1, in the generation of lung injury.

7.1 *Development of a suitable model*

A significant part of the early work in this thesis was the development of a reproducible, robust *in vivo* model of lung injury. A number of approaches were tried. The results are discussed in chapter 3. A model of reproducible injury was obtained by the instillation of endotoxin under direct visualisation. This model has a number of advantages: it generates a pattern of lung injury similar to that seen in humans; it has a similar time course with an early injury in response to the insult and resolution over time; by being done by direct visualisation of tracheal intubation the investigator can be confident that the insult has reached the tracheo-bronchial tree and that contamination of the instillate by oropharyngeal contents does not occur and finally by relying on the inhalation of the agent from the cannula by the animal the investigator can be confident that there is no barotrauma associated with the instillation of the insult. It is not known if this model reproduces the latter features of ARDS in humans as there was no histological evidence of fibrosis for example seen at any timepoint. It would be valuable to extend the timecourse of the model through to resolution of the injury. There are suggestions in the literature that the bleomycin model of pulmonary fibrosis is actually a good model of ARDS as it results in inflammatory cell damage and in some animals fibrosis [195]. The investigator is therefore faced with a choice. If they are interested in the early, neutrophil driven injury phase then endotoxin does represent a good model. However, if the area of interest focuses more on the resolution and reparative phases then other models may be of more interest.

The lack of injury seen with the other approaches is puzzling as they are reported to cause injury in the hands of other investigators. This may reflect species differences

[65], strain differences [157;163;255;256] or other unrecognised technical considerations.

7.2 MH-S data

Macrophages are known to secrete inflammatory mediators in response to LPS. In this thesis the generation of one of these, nitric oxide has been studied in detail. It has been shown that there is a significant production of NO in response to LPS and that the production of NO can be manipulated, as would be expected, by manipulating the concentration of the key substrate, oxygen, in the culture conditions. Of clinical relevance it has been shown that in conditions of substrate limitation (i.e. hypoxic conditions) there is still generation of the key enzyme iNOS and that it is active and when there is restoration of substrate it is rapidly able to generate large quantities of NO. This raises the possibility that in critically injured patients the resuscitation process acts as a second hit generating increased amounts of NO both by the action of increased delivery of oxygen to macrophages as a result of increasing the inspired fraction of oxygen and also by increasing the delivery of primed macrophages in unventilated areas of lung by alveolar recruitment manoeuvres in ventilated patients.

The presence of the three isoforms of NO Synthase in the MH-S cell line has been shown for the first time. This raises questions about the role of the lower output isoforms eNOS and nNOS in macrophages and is an area requiring further study.

The ability of DMOG to augment the NO response to LPS but reduce the secretion of TNFα is also worthy of further work. Other investigators [217;219] have shown that in models of gut inflammation that DMOG attenuates the inflammatory damage seen.

The contrasting effects on NO and TNFα release by alveolar macrophages in response to DMOG raises the possibility that in the presence of live bacteria DMOG may be able to increase the killing of the bacteria by NO dependent mechanisms but avoid some of the unnecessary surrounding inflammatory damage generated by the release of TNFα. This effect may be particularly useful in enclosed body cavities such as the pleural space where deleterious results of inflammation (loculated fluid collections and pleural thickening) can occur despite adequate antibiotic therapy (complicated parapneumonic effusion) [257]. If further work finds that DMOG alters the conversion of mTNFα to solTNFα then the therapeutic effect of this finding would be less clear. Increasing the mTNFα may actually result in increased local damage to tissues as a result of an increase in the local concentration of biologically active molecules [223]. Alternatively a situation could be envisaged where the inhibition of release of solTNFα results in a reduction in the systemic release of TNFα from the inflammatory nidus reducing the systemic effects of inflammation. Whatever the result this is an area of promise for future work.

7.3 In vivo Data

A key aim of this thesis was to test the role of myeloid cell Hif-1α in the development of Acute Lung injury in the animal model. Cramer et al [89] in their original report described dramatic reductions in the inflammatory response to stimuli in several models of tissue injury. However, in the data shown in this thesis there is less convincing evidence of a central role of Hif-1α in mediating injury in response to LPS. Other authors have shown that LPS increases Hif-1α in myeloid cells ([142;208]. The data presented here shows that quantitatively, using standard measures of lung injury, the reduction of myeloid cell Hif-1α in this animal line does not alter the pattern of acute injury following LPS instillation. In agreement with the

in vitro data there is a qualitative difference in the injury seen with evidence of reduced protein nitrosylation. It may be that the activation of other pathways, controlled by alternative transcription factors such as NF-κB, are still able to result in the activation of the inflammatory cascade. This possibility is supported by subsequent work from Professor Johnson's laboratory [218] where in a model of soft tissue infection there was no difference in inflammatory infiltrate in wild type or Hif-1α myeloid deplete animals. Alternatively there could be a dose effect and that at lower doses of LPS a difference in the inflammatory injury would have been seen. However as this thesis was interested in the clinical picture of ARDS it was felt appropriate to study the injury resulting from a significant insult. Time and animal constraints prevented a comparison of different doses of LPS at several different timepoints and it was felt that the initial study of high dose LPS over a clinically relevant timecourse was appropriate. Future work should involve following the resolution of the lung injury to see if the difference in BAL protein at 5 days persists and whether there are any clinically important differences in healing as a result of abnormal myeloid cell Hif-1α. Alterations in tissue repair are possible as Hif-1α is a key transcription factor controlling the release of CXCL-12 (also known as Stromal derived factor-1) which has been shown to be important in wound repair and progenitor cell trafficking [205;258]. The possibility of altered reparative processes also raises the possibility of altered disease in more chronic models of lung injury such as bleomycin induced lung fibrosis, an area of active research in the CIR currently.

7.4 Conclusion

Hypoxia inducible factor-1 is a transcription factor that is central to the cellular response to hypoxia. More recent work has shown that it is also modulated by hormones and cytokines involved in the inflammatory response. It is also increased in response to bacterial cell wall products (LPS). Controlling the expression of over 70 recognised gene products including a number of metabolic, inflammatory and angiogenic factors the role of Hif-1 in inflammatory injury has been the focus of recent interest.

This thesis has sought to study the role of Hif-1α in inflammatory lung injury with a specific focus on the role of myeloid cell Hif-1α. Quantitatively there appears to be little difference in lung injury as a result of Hif-1α depletion. However *in vivo* and *in vitro* data suggest that there are qualitative differences which may be of significance in the pathophysiology of Acute Lung Injury. These data suggest that further important insights may be obtained by the continued study of the role of hypoxia inducible factor-1 (and its family members) in models of lung injury.

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